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(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 461 438 B1

(12) # EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:
27.12.2006 Bulletin 2006/52

(21) Application number: **02758655.1**

(22) Date of filing: **20.09.2002**

(51) Int Cl.:
C12N 15/82 *(2006.01)* ***C12N 15/62*** *(2006.01)*
C12N 15/10 *(2006.01)* ***C07K 14/395*** *(2006.01)*
A01H 5/00 *(2006.01)*

(86) International application number:
PCT/GB2002/004293

(87) International publication number:
WO 2003/025172 (27.03.2003 Gazette 2003/13)

(54) **GENE EXPRESSION CONSTRUCT OF HAP1 ADAPTED FOR PLANT CELLS**

GEN-EXPRESSIONSKONSTRUKT VON HAP1 MODIFIZIERT FÜR PLANZENZELLEN

CONSTRUCTION D'EXPRESSION DE GENES

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR IE IT LI LU MC NL PT SE SK TR

(30) Priority: **21.09.2001 GB 0122828**

(43) Date of publication of application:
29.09.2004 Bulletin 2004/40

(60) Divisional application:
06005798.1 / 1 728 869

(73) Proprietor: **CAMBRIDGE UNIVERSITY TECHNICAL SERVICES LIMITED**
Cambridge, Cambridgeshire CB2 1TS (GB)

(72) Inventors:
- **HASELOFF, Jim**
  Comberton,
  Cambridgeshire CB3 7ED (GB)
- **BAUCH, Marion**
  3993 TP Houten (NL)
- **BOISNARD-LORIG, Corinne**
  69007 Lyon (FR)
- **HODGE, Sarah**
  Cambridge,
  Cambridgeshire CB2 7LG (GB)
- **LAPLAZE, Laurent**
  34000 Montpellier (FR)
- **RUNIONS, John**
  Cambridge,
  Cambridgeshire CB4 1NR (GB)
- **KURUP, Smita**
  Cambridge,
  Cambridgeshire CB5 8DJ (GB)

(74) Representative: **Kremer, Simon Mark et al**
Mewburn Ellis LLP
York House,
23 Kingsway
London WC2B 6HP (GB)

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Printed by Jouve, 75001 PARIS (FR)

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Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

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Description

TECHNICAL FIELD

[0001] The present invention relates generally to methods and materials for use in achieving and detecting gene expression, particularly localised expression of genes in a plant, and the detection of gene expression in a plant.

PRIOR ART

[0002] Developing multicellular tissues or organs generally demonstrate a capacity for self-organisation. For example, wounded tissues generally respond in a robust and coordinated fashion to allow repair, and local induction events can initiate prolonged and coordinated developmental processes. These types of developmental plasticity and functional autonomy are particularly evident in plant tissues. The basic features of a plant's body plan are established during embryogenesis, however its final form results from the continued growth of meristems and the formation of organs throughout its life, often in a modular and indeterminate fashion. Plant cells are constrained by rigid cell walls and are generally non-motile, so there is the clear possibility that cell fates within a meristem are determined by lineage. However, evidence from plant chimera and wounding studies have demonstrated a more important role for cell-cell interactions during fate determination (reviewed in Steeves & Sussex, Patterns in Plant Development, 1989) and laser ablation of cells within the *Arabidopsis* root meristem has shown that after the death of a cell, a neighbouring cell can be triggered to divide and compensate for the loss (van der Berg et al., Nature 378:62-65, 1995). It is likely that positional information during plant development is obtained via cell-cell contact, and that the coordination and fate of cells within a developing meristem may be determined by a network of local cellular interactions. The present inventors have chosen the *Arabidopsis* root meristem as a model system for investigating intercellular interactions. The root meristem possesses indeterminate growth and has a simple and transparent architecture. *Arabidopsis* is genetically amenable, and one can routinely generate transgenic lines for work with the intact organism.

[0003] However, in order to dissect and engineer local cell-cell interactions, it is crucial that one can (i) clearly visualise individual cells inside living meristems and (ii) have the means to perturb them. Over the past several years, the present inventors have developed a set of genetic and optical techniques which enable the manipulation and visualisation of cells within living plants.

[0004] In order to genetically manipulate cells during meristem development, the inventors have previously devised a scheme for targeted gene expression, which is based on a method widely used in *Drosophila* (Brand and Perrimon, Development 118:401-415, 1993). PCT/GB97/00406 describes a method using a highly modified transcription factor derived from the yeast GAL4 protein to form Arabidopsis plant lines that display localised expression of the foreign transcription factor, which can be used to trigger the ectopic expression of any other chosen gene at a particular time and place during the growth of the plant. The expression of the transcription factor can be followed using GFP as a reporter gene.

[0005] However, this system has a number of problems, one of which that it is limited in its application to the activation of a single chosen gene or the simultaneous activation of different genes within the same cell types, but does not allow the activation of different genes in different cell types and/or at different times within the same plant.

[0006] Thus it can be seen that another modified transcription factor which could be used in conjunction with or as an alternative to GAL4 would provide a valuable contribution to the art.

[0007] In order to visualise plant cells in transgenic plants, the gene encoding jellyfish green fluorescent protein (GFP) has been adapted for use as a reporter gene. The wild-type GFP cDNA is not expressed in *Arabidopsis.* The present inventors have extensively modified the gfp gene to remove a cryptic intron, to introduce mutations that confer improved folding and spectral properties and to alter the subcellular localisation of the protein. All of these alterations have been incorporated into a single modified form of the gene *(mgfp5-ER)* which can be routinely used for monitoring gene expression and marking cells in live transgenic plants (Siemering et al., Current Biology 6:1653-1663, 1996; Haseloff et al., PNAS 94:2122-2127,1997).

[0008] Fluorescence microscopy techniques for high resolution observation of living cells have been developed. The expression of GFP within an organism produces an intrinsic fluorescence that colours normal cellular processes, and high resolution optical techniques can be used non-invasively to monitor the dynamic activities of these living cells. Using coverslip-based culture vessels, specialised microscope objectives and the optical sectioning properties of the confocal microscope, it is possible to monitor simply and precisely both the arrangement of living cells within a meristem, and their behaviour through long time-lapse observations. Further, the present inventors have recently constructed cyan and yellow emitting GFP variants that can be distinguished from the green fluorescent protein during confocal microscopy. These colour variants have enabled simultaneous imaging of different tagged proteins in living cells (Haseloff, J., "GFP variants for multispectral imaging of living cells", in Methods in Cell Biology, Vol. 58, Kay, S. and Sullivan, K. Eds. Academic Press (1999).

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[0009] However, the usefulness of reporter proteins such as GFP is limited by the fact that, when plant tissues are cleared and stained for detailed 3-Dimensional analysis, reporter proteins such as GFP are lost from the tissues.

[0010] Thus it can be seen that a robust insoluble reporter protein would provide a valuable contribution to the art.

[0011] Pfeifer K et al: "Functional dissection and sequence of yeast HAP1 activator" (Cell, vol. 56, no. 2, 1989, pages 291-302) presents the DNA sequence of the yeast activator HAP1 and comments on its functional domains.

[0012] Defranoux Nadine et al: "Functional analysis of the zinc cluster domain of the CYP1 (HAP1) complex regulator in heme-sufficient and heme-deficient yeast cells" (Molecular & General Genetics, vol. 242, no. 6, 1994, pages 699-707) also discusses HAP1, particularly with respect to oxygen and heme regulation.

[0013] Todd Richard B et al: "Evolution of a fungal regulatory gene family: The Zn(II)2Cys6 binuclear cluster DNA binding motif" (Fungal Genetics and Biology, vol. 21; no. 3, 1997., pages 388-405) describes the evolutionary relationship of several fungal regulatory genes.

[0014] WO97 41228 A (Pioneer Hibred International, Inc.; Gordon-Kamm W et al) 6 November 1997 (1997-11-06) discusses the uses of GFP and a screenable marker for plant transformation.

[0015] sommer-Knudsen J et al: 'Hydroxyproline-rich plant glycoproteins' (Phytochemistry, Pergamon Press, GB, vol. 47, no. 4, February 1998 (1998-02), pages 483-497) is a review of the four major groups of these glycoproteins, and the similarities and differences between them.

DISCLOSURE OF THE INVENTION

[0016] The present invention utilises an isolated nucleic acid, expressible in a plant cell, encoding at least an effective portion of a HAP1 DNA-binding domain, wherein the sequence has an A/T base content substantially reduced compared to the wild-type sequence.

"Effective portion"

[0017] An effective portion of the DNA-binding domain is a portion sufficient to retain most (i.e. over 50%) of the DNA-binding activity of the full length DNA-binding domain. Preferably the "effective portion" comprises amino acid residues 1 to 94 of the yeast polypeptide, which we have found to be the minimal amount required to retain DNA binding activity. Typically, the "effective portion" will comprise at least 60% of the full-length sequence of the DNA-binding domain.

"A/T base content"

[0018] the A/T content of the wild-type yeast sequence encoding the DNA-binding domain of HAP1 is about 54%. The %A/T base content of the sequence of the invention encoding the effective portion of the HAP1 should be taken to be substantially reduced when it is less than 45%. Preferably, it will be less than 40%, most preferably it is 39%.

[0019] Preferably, the encoded polypeptide will have the identical amino acid sequence to the wild-type HAP1 polypeptide shown in Figure 2b (bottom line). However, on the other hand, the encoded polypeptide may comprise an amino acid sequence which differs by one or more amino acid residues from the amino acid sequence shown in Figure 2b (bottom line).

[0020] Nucleic acid encoding at least an effective portion of a HAP1 DNA-binding domain which is an amino acid sequence mutant, variant or derivative of the amino acid sequence shown in Figure 2b, wherein the nucleic acid sequence has an A/T base content substantially reduced compared to the wild-type sequence is therefore included within the scope of the present invention.

[0021] A peptide which is an amino acid sequence variant, derivative or mutant of an amino acid sequence of a peptide may comprise an amino acid sequence which shares greater than about 60% sequence identity with the sequence of the amino acid sequence shown in Figure 2b (bottom line), greater than about 70%, greater than about 80%, greater than about 90% or greater than about 95%. The sequence may share greater than about 70% similarity, greater than about 80% similarity, greater than about 90% similarity or greater than about 95% similarity with the amino acid sequence shown in Figure 2b (bottom line).

[0022] For amino acid "homology", this may be understood to be similarity (according to the established principles of amino acid similarity, e.g. as determined using the algorithm GAP (as described below) or identity.

[0023] Amino acid similarity is generally defined with reference to the algorithm GAP (Genetics Computer Group, Madison, WI). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, the default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4. Use of GAP may be preferred but other algorithms may be used, e.g. BLAST (which uses the method of Altschul et al. (1990) J. Mol. Biol. 215: 405-410), FASTA (which uses the method of Pearson and Lipman (1988) PNAS USA 85: 2444-2448), or the Smith-Waterman algorithm (Smith and Waterman (1981) J. Mol Biol. 147: 195-197), generally employing default parameters.

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[0024] Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine.

[0025] In a first aspect, the nucleic acid of the invention comprises the nucleic acid sequence of modified HAP1 (labelled mHAP1 - middle line of Figure 2b), which sequence has a substantially reduced A/T content relative to the wild-type yeast sequence which is shown in the top line of Figure 2b, labelled HAP1 (substantially reduced base content is defined above).

[0026] The nucleic acid encoding the portion of HAP1 binding domain is fused to a nucleic acid sequence, said sequence encodes a transcriptional activator. The transcriptional activator may be the activation domain of the HAP1 protein, in which case the sequence encoding the HAP1 transcriptional activator should be optimised for expression in plants, by, for example, reducing the A/T content thereof. Alternatively, the transcriptional activator may be any transcriptional activator known by the skilled person to be active in plants. In such cases, the sequence of the invention thus encodes a chimeric polypeptide. In a preferred embodiment, the transcriptional activator domain is that of the herpes simplex virus (HSV) VP-16 (Greaves and O'Hare, J. Virol 63 1641-1650, (1989)). Preferably, the sequence comprises the nucleic acid sequence of VP16 shown in Figure 2c (from nucleotide 293 onwards, i.e., the part of the top line of sequence which is in upper case). Thus in a preferred embodiment of the invention, there is provided a chimeric polypeptide comprising the nucleic acid sequence of the mHAP1-VPI6 chimera shown in Figure 2c (i.e., the entire nucleotide sequence of Figure 2c (top line)).

[0027] Other suitable transcriptional activation domains include certain peptides encoded by the E.coli genomic DNA fragments (Ma and Ptashne, Cell 51 113-119 (1987)) or synthetic peptides designed to form amphiphilic α-helix. (Giniger and Ptashne Nature 330 670-672 (1987)). A common requirement for suitable transcriptional activation domains is the need for excess charge (Gill and Ptashne, Cell 51 113-119 (1987), Estruch et al Nucl.Acids Res. 22 3983-3989 (1994)). Using this criteria, the skilled person is able to select or synthesise sequences which encode transcriptional activation activity in plants.

[0028] In a further aspect of the present invention, there is provided a nucleic acid construct, comprising the nucleic acid defined above.

Preferred Vectors

[0029] In one aspect of the present invention, the nucleic acid construct is in the form of a recombinant and preferably replicable vector. "Vector" is, defined to include, inter alia, any plasmid, cosmid, phage or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform a prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

[0030] Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press or Current Protocols in Molecular Biology, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992.

[0031] The nucleic acid in the vector is under the control of, and operably linked to, an appropriate promoter or other regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial, or plant cell. The vector may be a bi-functional expression vector which functions in multiple hosts.

[0032] By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA).

[0033] "Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

[0034] Thus this aspect of the invention provides a gene construct, preferably a replicable vector, comprising a promoter operably linked to a nucleic acid provided by the present invention i.e. the sequence encoding the HAP1 DNA-binding domain. The sequence has an A/T base content substantially reduced compared to the wildtype sequence.

[0035] Particularly of interest in the present context are nucleic acid constructs which operate as plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148). Suitable vectors may include plant viral-derived vectors (see e.g. EP-A-194809).

[0036] The promoter to be used in the construct is an "enhancer dependent" (or naive) promoter, which requires the presence of a suitable enhancer sequence and appropriate transcription factors to cause substantial levels of transcription. Such naive promoters correspond to the TATA box region of known plant promoters. "Plant promoters" should be

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understood to refer to promoters (e.g. viral or bacterial) active in a plant cell. The naive promoter is in competent relationship with the sequence encoding the HAP1 DNA binding domain and transcription activation domain such that if the promoter is inserted into a plant host cell genome in functional relationship with an enhancer sequence and required transcription factors, the promoter will direct expression of the HAP1 DNA-binding domain in a tissue specific manner.

Reporter Genes '

[0037] In preferred embodiments of the invention, a reporter gene operably linked to a HAP1 upstream activation sequence (UAS) is provided, such that the reporter gene will be expressed in response to synthesis of the transcriptional activator discussed above. The reporter gene may be present as part of a nucleic acid construct comprising the nucleic acid encoding an effective portion of a HAP1 DNA-binding domain. Alternatively, the reporter gene may be present in another nucleic acid construct.

[0038] The reporter gene may be any suitable reporter gene known to the skilled person as being active in plants. Use of a reporter gene facilitates determination of the UAS activity by reference to protein production. The reporter gene preferably encodes an enzyme which catalyses a reaction which produces a detectable signal, preferably a visually detectable signal, such as a coloured product. Many examples are known, including β-galactosidase and luciferase. β-galactosidase activity may be assayed by production of blue colour on substrate, the assay being by eye or by use of a spectro-photometer to measure absorbance. Fluorescence, for example that produced as a result of luciferase activity, may be quantitated using a spectrophotometer. Radioactive assays may be used, for instance using chloramphenicol acetyltransferase, which may also be used in non-radioactive assays. The presence and/or amount of gene product resulting from expression from the reporter gene may be determined using a molecule able to bind the product, such as an antibody or fragment thereof. The binding molecule may be labelled directly or indirectly using any standard technique.

[0039] Those skilled in the art are well aware of a multitude of possible reporter genes and assay techniques which may be used to determine UAS activity. Any suitable reporter/assay may be used, and it should be appreciated that no particular choice is essential to or a limitation of the present invention.

[0040] In preferred embodiments of the invention, the reporter gene is GFP. The gfp gene may be the wild-type *Aequorea* victoria gene, or may be modified in any conventional way. For example, in a preferred embodiment, the gfp gene is *mgfp5-ER,* which has a cryptic intron removed, and has mutations which confer improved folding and spectral properties and altered subcellular localisation of the protein (Siemering et al., Current Biology 6:1653-1663, 1996; Haseloff et al., PNAS 94:2122-2127,1997).

[0041] However, although GFP (wild-type and modified forms) provides a convenient method of marking cells, clearing and staining of plant tissues normally result in loss of the green fluorescent protein from the tissues. For example, after treatment with even gentle clearing agents such as 50% ethanol or 50% glycerol, the protein becomes dislodged from treated cells.

[0042] The present inventors have overcome this problem by developing a new robust surface marker for visualisation of plant cells utilising GFP fused to an extensin protein(see below). Thus, in preferred embodiments of the invention, the reporter gene comprises the GFP extensin reporter gene fusion as described below.

Host Plants

[0043] In a further aspect of the invention, the invention provides a plant, plantlet or part thereof (e.g. a plant host cell or cell line) comprising a nucleic acid construct of the invention.

[0044] In preferred embodiments, the construct will have become stably integrated into a plant cell genome. In particular, the invention provides a plurality of plants, plantlets or parts thereof comprising a library, each plant or part thereof comprising a stably maintained nucleic acid sequence encoding an effective portion of the HAP1 DNA-binding domain as defined above. Preferably, the nucleic acid construct will be incorporated into the genome of plant cells present in the library. The library may be of any plant of interest to the skilled person. Suitable plants include maize, rice, tobacco, petunia, carrot, potato and Arabidopsis. Where the term "plant" is referred to hereinafter, unless context demands otherwise, it will be understood that the invention applies also to plantlets or parts of plants.

[0045] Each plant, plantlet or part thereof may have a particular pattern of expression of the integrated reporter gene. Thus, introduction of a further gene, having a HAP1-responsive UAS into the cells will result in the expression of the introduced gene in the same temporal/spatial pattern as the reporter gene, enabling expression of a gene of interest in selected tissues and/or at selected times.

Uses of Constructs of the Invention

[0046] In a preferred embodiment of the invention, the nucleic acid construct can be used in an "enhancer trap assay"

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to identify plant enhancer sequences (Sundaresan et al, Genes and Dev. 9 1797-1810). In such cases, the nucleic acid construct will preferably comprise right and left Ti-DNA, to enable random, stable insertion into the genome of a plant host cell.

[0047] As well as nucleic acid constructs for use in "enhancer trap assays", the invention further provides a method of identifying a plant "enhancer" nucleic acid sequence, comprising the steps of:

transforming a plant cell host with a nucleic acid construct comprising a naive promoter sequence and a sequence encoding an effective portion of an HAP1 binding domain fused to a transcription activating domain, under the control of said naive promoter sequence,

wherein, when said plant is transformed with said construct such that the promoter is in functional relationship with a host cell enhancer sequence, the promoter will direct expression of said HAP1 binding domain operably linked to a transcription activating domain in the presence of "enhancer" transcription factors.

[0048] Thus the expression of said HAP1 binding domain operably linked to a transcription activating domain will indicate the presence of such an "enhancer" sequence. Optionally, the method includes the further step of identifying the position and nucleic acid sequence of the enhancer sequence.

[0049] For example, this may be performed by standard inverse PCR (I-PCR) or TAIL -PCR amplification of flanking sequences (see Sambrook & Russell, Molecular Cloning: a laboratory manual. 3rd edition, CSHL press 2001: sections 4.75 (TAIL-PCR), 8-81 (I-PCR)).

[0050] The nucleic acid construct of the invention may thus also be used to control expression of an heterologous gene in a plant or part thereof. Thus in a further aspect of the present invention, there is provided a method of controlling expression of a gene of interest in a plant or part thereof comprising the steps of:

introducing the gene of interest into a plant or part thereof, said gene of interest having an HAP1 responsive upstream activation sequence,
said plant or part thereof comprising a nucleic acid sequence encoding an effective portion of an HAP1 binding domain fused to a transcription activating domain, under the control of said naive promoter such that expression of a transcriptional activator from said sequence is limited to those cell types in which a naive promoter sequence is in functional relationship with a host cell enhancer sequence;

wherein binding of said transcriptional activator to said upstream activator sequence causes transcriptional activation of the gene of interest.

[0051] The nucleic acid of the invention enables the activation of different genes of interest in different cell types and/or at different times within the same plant, particularly wherein the nucleic acid encoding at least an effective portion of a HAP1 DNA-binding domain is used in conjunction with nucleic acid encoding a modified GAL4 transcription factor, for example that described in WO97/30164, the expression of each gene of interest being under the operable control of a different transcription factor.

[0052] Thus, included within the scope of the present invention is a method of independently controlling expression of a first and a second gene of interest in a plant comprising the steps of:

introducing the first gene of interest into a plant or part thereof, said first gene of interest having an HAP1 responsive upstream activation sequence;
introducing the second gene of interest into a plant or part thereof, said second gene of interest having a GAL4 responsive upstream activation sequence;
said plant or part thereof comprising a first nucleic acid sequence, which encodes a HAP1 transcriptional activator and a second nucleic acid sequence, which encodes a GAL4 transcriptional activator;

wherein binding of said HAP1 transcriptional activator to said upstream activator sequence causes transcriptional activation of the first gene of interest and binding of said GAL4 transcriptional activator to said upstream activator sequence causes transcriptional activation of the second gene of interest.

[0053] In this way, where the expression of the HAP1 transcriptional activator and the GAL4 transcriptional activator are each independently under the control of a different naive promoter sequence, expression of each transcriptional activator is limited to those cell types in which the naive promoter sequence is in functional relationship with a host cell enhancer sequence.

[0054] Moreover, using the nucleic acid construct of the invention, the simultaneous expression of a number of genes of interest may be controlled. Thus, in a further aspect of the invention, there is provided a method of co-ordinating the expression of a plurality of genes of interest in a plant or part thereof, comprising the steps of introducing the genes of interest into a plant or part thereof,

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said genes of interest each being under the control of an HAP1 responsive upstream activation sequence and said plant or,part thereof comprising a nucleic acid sequence of the invention capable of expressing an HAP1 transcriptional activator,
wherein binding of said HAP1 transcriptional activator to said upstream activator sequence causes transcriptional activation of the genes of interest.

[0055] The plurality of genes may all be associated with a single UAS, which facilitates their introduction into the plant or part thereof. Alternatively, one or more genes may be operably linked to a respective UAS.

[0056] Using the nucleic acid construct of the invention in conjunction with a nucleic acid construct encoding a GAL4 transcriptional activator, the expression of a first group of genes may be coordinated and the expression of a second group of genes may be co-ordinated, wherein each group of genes is expressed in different cell-types and/or at different times.

[0057] The gene or genes of interest may be any target gene or genes, the expression of which the researcher wishes to study. In preferred embodiments, the gene or genes of interest may be developmental genes or may encode one or more toxins. For example, a gene of interest may encode a toxin such as the A-chain of diphtheria toxin (DTA) and thus the method may be used to kill specific cells , for example, within the root meristem. Other genes of interest may encode one or more cell cycle regulatory proteins, in which case expression of such gene or genes may be used to drive misexpression of such proteins and activate or inhibit particular cell divisions e.g. within the root meristem. The gene or genes of interest may encode homeodomain proteins and thus the effect of their ectopic expression on cell fate determination may be studied.

[0058] The gene of interest may be of unknown function. Using the methods of the invention, the function of a gene of interest may be determined by comparing the phenotype of plants, or parts thereof in which the gene of interest is expressed with the phenotype of plants or parts thereof in which it is not expressed. Thus the invention extends to a method of determining the function of a gene of interest comprising the steps of:

introducing agene of interest into a plant or part thereof, said gene of interest having an HAP1 responsive upstream activation sequence;
said plant or part thereof comprising a nucleic acid sequence, which encodes a HAP1 transcriptional activator;

wherein binding of said HAP1 transcriptional activator to said upstream activator sequence causes transcriptional activation of the gene of interest;
comparing the phenotype of said plant or part thereof in which said gene of interest is expressed with a second plant or part thereof in which said gene of interest is not expressed.

[0059] The gene or genes of interest may be "introduced" into the plant or part thereof using any conventional technique, for example, using any one of the vectors described above. Conveniently, the gene of interest is introduced using Agrobacterium mediated transformation.

[0060] In preferred embodiments of the methods of the invention, a reporter gene having an HAP1 responsive upstream activation sequence is provided, such that binding of said transcriptional activator to said upstream activator sequence causes transcriptional activation of the reporter gene. The reporter gene may be any suitable reporter gene, details of which are given above. Preferably, the reporter gene will be the extensin-GFP fusion gene described below.

Extensin-GFP Reporter Gene Construct

[0061] As described above, conventional cell markers utilising GFP suffer from the disadvantage that, during clearing of the tissues, the GFP is often lost to at least some degree. The present inventors have overcome this problem by developing a new robust surface marker for visualisation of plant cells utilising GFP.

[0062] This embodiment of the invention is based on the inventors' demonstration that, when a coding sequence encoding GFP is fused to the coding sequence of the carrot extensin gene, the resulting expressed extensin-GFP fusion protein results in a bright marker resistant to clearing techniques which normally result in complete loss of GFP from treated tissues. Thus in a preferred embodiment of the present invention, the reporter gene construct is an extensin-GFP reporter gene.

[0063] Thus, this embodiment of the present invention utilises a gene fusion, expressible in a plant cell, comprising a nucleic acid sequence encoding a green fluorescent protein operably linked to a nucleic acid sequence encoding at least an effective portion of extensin.

[0064] An effective portion of extensin is a portion sufficient to retain most (i.e. over 50%) of the activity of the full length carrot extensin. Typically, the "effective portion" will comprise at least 60% of the full-length sequence of the carrot extensin. Wild-type extensin is involved in cell wall expansion in plants, other cell wall expansion proteins may be used, as would be understood by the person skilled in the art.

[0065] In a preferred embodiment, the nucleic acid sequence encoding the green fluorescent protein is the nucleic

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acid sequence of GFP shown in Figure 3b (uppercase nucleic acid sequence) and/or the nucleic acid sequence encoding the extensin protein is the nucleic acid sequence of extensin shown in Figure 3b (lowercase amino acid sequence). The gene fusion preferably comprises the a nucleic acid molecule having the entire sequence shown in Figure 3b.

[0066] Thus, the invention provides the use of the extensin-GFP gene fusion of the invention in the "enhancer trap" assay described above. In such an assay, the extensin-GFP gene fusion is in operable relationship with an upstream activation sequence or promoter, the activation of which by an activation domain of an HAP1 binding domain operably linked to a transcription activating domain causes expression of the extensin-GFP gene fusion and thus enables the visualisation of expression of the HAP1 transcription factor.

Screening for Reporter Gene Expression in Plants

[0067] Expression of a reporter gene may be monitored using any suitable technique known to the person skilled in the art. For example, for the screening of shoots and roots of transgenic plantlets in which the reporter protein is a fluorescent protein such as GFP or the extensin-GFP of the invention, expression can be screened directly using epif-luorescence microscopy, to, for example, monitor expression in developing meristems.

[0068] For the monitoring of expression of fluorescent proteins, multispectral dynamic imaging may be used. Such confocal microscope based methods allow high resolution observation of living cells. The expression of GFP within an organism produces an intrinsic fluorescence that colours normal cellular processes, and high resolution optical techniques can be used, non-invasively to monitor the dynamic activities of these living cells. Using coverslip-based culture vessels, specialised microscope objectives and the optical sectioning properties of the confocal microscope, it is possible to monitor simply and precisely both the arrangement of living cells within a meristem, and their behaviour through long timelapse observations (see http://www.plantsci.cam.ac.uk/Haseloff). Further, the use of cyan and yellow emitting GFP variants that can be distinguished from the green fluorescent protein during confocal microscopy enable simultaneous imaging of different tagged proteins in living cells.

[0069] As a further or alternative screen, a second screen may be used on adult transgenic plants, in which parts of the plants such as the flowers or siliques are dissected and the fluorescence of parts monitored. Such screens are particularly useful for identifying expression patterns in embryos and floral parts, in which GFP may not be expressed in plantlets.

[0070] The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

FIGURES

[0071]

Fig 1 shows oligonucleotides for construction of mHAP1 DNA binding domain.

Fig 2a shows a schematic diagram of the mHAP1 -VP16 synthetic transcription activator chimeric gene

Fig 2b shows the nucleic acid sequence of wild-type HAP1 (top line - labelled HAP1); the nucleic acid sequence of modified HAP1 (middle line - labelled mHAP1); and the encoded amino acid sequence (bottom line)

Fig 2c shows, the nucleic acid sequence (top line) of the mHAP1 - VP16 synthetic transcription activator chimeric gene, in which the HAP1 sequence is the modified sequence (running from the 5' terminus to position 292); the VP19 nucleic acid sequence runs from position 293 onwards and is shown in upper case); and the amino acid sequence of the synthetic transcription activator chimeric protein is shown in the bottom line.

Fig 3a shows a schematic diagram of the extensin-GFP gene fusion.

Fig 3b shows the coding sequence of the extensin-GFP fusion of Fig 3a in the top line and the encoded amino acid sequence in the bottom line. Extensin nucleotide sequence is shown in lower case and the GFP nucleotide sequence is shown in uppercase.

Figure 4 shows expression of an extensin-GFP gene fusion in transgenic Arabidopsis. A 35S-extensin-GFP construction was introduced into Arabidopsis using Agrobacterium mediated transformation. Confocal optical sections of transformed plantlets are shown. Chlorophyll autofluorescence is seen in the red channel.

Figure 5 shows oligonucleotides used in construction of a HAP1 DNA binding site.

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EXAMPLES

Example 1 Construction of the modified HAP1-VP16 gene

[0072] The yeast HAP1 protein is a member of a family of zinc-finger (Cys_4) transcription factors which are limited to fungi, and homologues have not been found in plants to date. Yeast genes have a high A/T content and are often poorly expressed in *Arabidopsis* due to aberrant post-transcriptional processing. A synthetic gene which has an elevated G/C content, and in which the DNA binding domain is fused to the highly active and G/C-rich transcription activator domain of VP16, was constructed:

[0073] Three long oligonucleotides, mHAP1-A, B & C (shown in Figure 1) were made using automated synthesis on solid supports. The oligonucleotides encoded the predicted DNA binding domain of HAP1 protein with modified codon usage. Codon usage was modified according to the following criteria:

(i) GC content was increased
(ii) splice junction consensus sequences were avoided
(iii) the resultant amino acid sequence encoded by the nucleic acid was unchanged.

[0074] Oligonucleotides mHAP1-D and E (Figure 1) contained complementary sequence corresponding to the junctions of the three longer oligonucleotides. The synthetic oligonucleotides were purified by polyacrylamide gel electrophoresis and phosphorylated after incubation with ATP and T4 polynucleotide kinase. The oligonucleotides were then mixed and heated at 94°C for 1 min and annealed at 60°C for 5 min. After cooling, the sample was treated with T4 DNA ligase to produce a small quantity of single-strand DNA corresponding to the mHAP1 DNA binding domain. This was then used as a template for PCR amplification with oligonucleotides mHAP1-5' and 3' (Figure 1) .

[0075] Figure 2a shows in diagrammatic form the mHAP1 -VP16 synthetic transcription activator chimeric gene (Figure 2c entire sequence). The DNA sequence, encoding, in the 5' portion (bases 1 to 292), the modified HAP1 DNA binding domain (see also Figure 2b middle sequence) and encoding, in the 3' portion (bases 293 - 533), the transcriptional activation domain from HSV VP16, is shown as the top sequence of Figure 2c with the encoded amino acid sequence shown below. The SacI restriction endonuclease site within the gene is marked.

[0076] The wild-type sequence of the HAP1 binding domain (see the top sequence of Figure 2b) is shown above for comparison of the A/T%. The wild-type HAP1 DNA binding domain DNA sequence is A/T rich.

Example 2 Construction of Insoluble GFP marker.

[0077] A variant of GFP was fused to the coding sequence of a carrot extensin. PCR amplification was used to obtain a copy of the extensin gene, isolated from carrots purchased in Cambridge market square. The carrot gene was genetically fused to a variant of green fluorescent protein obtained from Packard Biosciences (Meridian, Conneticut(GFPemd).

[0078] A schematic diagram of the extensin-GFP gene fusion is shown in Figure 3a with the coding sequence shown in Figure 3b (top line), the encoded amino acid sequence is shown below in the bottom line of Figure 3b. Extensin sequence is in lower case and GFP sequence is in uppercase.

Example 3 Expression of extensin GFP gene fusion in transgenic *Arabidopsis*

a) Construction of the 35S-extensin-GFP construct.

[0079] A variant of GFP was fused to the coding sequence of a carrot extensin. PCR amplification was used to obtain a copy of the gene, isolated from carrots purchased in Cambridge market square. The carrot gene was genetically fused to a variant of green fluorescent protein obtained from Packard Bioscience (GFPemd (emerald)). Expression of this gene fusion in transgenic Arabidopsis tissues results in the decoration of cell walls with bright fluorescence.

Construction of the GFP-extensin gene

[0080] The following oligonucleotides were synthesised, and used as primers for the PCR amplification of the carrot extensin gene,

CarExt5
GGC GGA TCC AAC AAT GGG AAG AAT TGC TAG AGG CTC

CarExt3

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GGC GGA TTC GTA GTG GTG AGG AGG AGG AGG TGA CGT

[0081] Template carrot DNA was isolated using a Qiagen DNA extraction kit (UK - QIAGEN Ltd., Boundary Court, Gatwick Road, Crawley, West Sussex, RH10 9AX), and 1 microgram of isolated carrot DNA was used in a PCR reaction with VENT polymerase (New England Biolabs), (30 cycles: 92°C 30sec, 60°C 30sec, 72°C 60sec). The amplified product was purified by 1% agarose gel electrophoresis, and digested with the restriction endonucleases *BamH1* and *EcoR1.* The cut fragment was then ligated into a plasmid vector that contained a GFP gene with an EcoR1 restriction fused to the N-terminus of the coding sequence, Haseloff, J., Siemering, K.R., Prasher, D.C. and Hodge, S *Removal of* a *cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly.* Proc. Natl. Acad. Sci. USA. 94, 2122-2127 (1997). The resulting plasmid contained a translational fusion between carrot extensin and the GFPemd gene (Packard Bioscience) .

[0082] Figure 3a shows a schematic diagram of the extensin-GFP gene fusion. The extensin sequences lie between the BamH1 and EcoR1 sites, and the GFP sequences lie between the EcoR1 and Sac1 sequences.

[0083] This reporter gene has been tested by insertion into plant transformation vectors, and expressed in transgenic Arabidopsis plants behind a constitutive CaMV 35S promoter, and as part of a HAP1-based enhancer trap vector. Bright, cell wall localised fluorescence results in both cases.

b) Transformation of *Arabidopsis* thaliana

[0084] Arabidopsis thaliana was transformed using the method given in PCT/GB97/00406.

[0085] Expression of the extensin-GFP gene fusion in transgenic *Arabidopsis* tissues results in the decoration of cell walls with bright fluorescence (Fig 4). Extensin becomes covalently linked to the cell wall matrix, and the GFP-extensin marker is resistant to various clearing techniques that normally result in complete loss of the protein from treated tissues. For example, the cell wall bound signal is retained after glycerol infiltration.

Example 4 Construction of a HAP1 promoter for use in plants

[0086] An optimised multimeric binding site for HAP 1 was synthesised and cloned behind a GFP promoter. Oligonucleotides used in the construction of the binding site are shown in Figure 5 (UASHAP1a and UASHAP1b).

[0087] The oligonucleotides were phosphorylated using polynucleotide kinase, annealed, and ligated into the *Hin*D III-*Xba* I sites of a UAS_{GAL4} containing vector. The oligonucleotide sequences replaced the UAS_{GAL4} with the appropriate UAS_{HAP1} sequences - already positioned upstream of a plant TATA box and GFP reporter gene.

Example 5 Construction of HAP1-GFP Enhancer Trap Vector

[0088] An enhancer trap vector was constructed using the modified HAP1-VP16 gene positioned with a minimal (naive) promoter and the extensin GFP gene fusion as described above.

[0089] The PCR product produced as described in Example 1 was cut with *BamH1 and SacI* restriction endonucleases and purified after electrophoresis through a 1.5% LGT agarose gel.

[0090] The plasmid pCMVGal65 (Cousens et al., EMBO J. 8:2337-2342, 1989) was used as a source of the *VP16* sequence. A *Sac*I-*Kpn*I fragment, which encodes the activation domain of the herpes simplex virus VP16 protein, had been previously fused to a modified form of the GAL4 DNA binding domain (Haseloff and Hodge, US patent 6,255,558 B1) within a plant enhancer-trap vector, pET-15 (GAL4-GFP). The GAL4 sequence was excised from the pET-15 vector by restriction endonuclease digestion with *BamH1* and *Sac1,* and replaced by ligation with the amplified mHAP1 sequence (see construction of extensin-GFP gene).

[0091] The *mHAP1-VP16* gene was directly assayed for activity in transformed *Arabidopsis* plants by Agrobacterium-mediated transformation (Valvekens et al. Proc. Natl. Acad. Sci. USA 85:5536-5540, 1988).

Example 6 Enhancer Trap Screen

[0092] The vector was used to transform *Arabidopsis* thaliana using Agrobacterium mediated transformation as described in Example 3. In this way, large numbers of transgenic calli are regenerated, induced to form roots and shoots and are directly screened by epifluorescence microscopy for extensin-GFP expression in the developing meristems.

[0093] A suitable protocol is as follows:

(1) 20-100 transgenic Arabidopsis seed were placed in a 1.5 ml microfuge tube and washed for about 1 min with 1 ml of ethanol.

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(2) Seeds were then incubated with 1 ml of a surface sterilising solution containing 1% (w/v) sodium hypochlorite and 0.1% (v/v) NP40 detergent, for 15 min at room temperature.

(3) The seeds were then washed three times with 1 ml of sterile water, and transferred by pipette to agar plates containing GM medium (Valvekens, D., Van Montagu, M and Van Lijsebettens, M. (1988) Agrobacterium tumefaciens-mediated transformation of *Arabidopsis* thaliana root explants by using kanamycin selection.

Proceedings of the National Academy of Sciences U.S.A. 85:5536-5540).

[0094]

1x Murashige and Skoog basal medium with Gamborgs B5 vitamins (Sigma)
1% sucrose
0.5 g/l 2-(N-morpholino)ethanesulfonic acid (MES)
0.8% agar
(adjusted to pH 5.7 with 1M KOH)
25 mg/l kanamycin was added if antibiotic selection of transgenic seedlings was necessary.

[0095] These procedures were performed in a laminar flow hood.

[0096] Alternatively, for extended timelapse imaging of roots, sterile seeds were sown in coverslip based vessels (Nunc) which comprised 4 wells, each containing about 400μl of low gelling temperature agarose with GM medium. The roots of these plants grow down though the media and then along the surface of the coverslip. The roots are then ideally positioned for high resolution microscopic imaging through the base of the vessel.

[0097] (4) Sealed plates or vessels were incubated for 1-3 days in the dark at 4°C, and then transferred to an artificially lit growth room at 23°C for germination.

[0098] (5) Arabidopsis seedlings germinate after 3 days, and can be used for microscopy for several weeks. Root and shoot tissues can be directly scored for GFP expression using an inverted fluorescence microscope (Leitz DM-IL) fitted with filter sets suitable for UV (Leitz-D; excitation filter 355-425 nm, dichroic mirror 455 nm, longpass emission filter 460 nm) and blue (Leitz-I3; excitation filter 450-490 nm, dichroic mirror 510 nm, longpass emission filter 520 nm) light excitation of GFP. Roots, which grow along the base of the petri dish can be observed directly by epifluorescence microscopy through the clear plastic base. Shoot tissues were directly observed in inverted dishes by using one or two 7mm threaded extension tubes with a 4x objective (EF 4/0.12), that gave greater working distances. Epifluorescence images were captured in Adobe Photoshop using a Sony DXC-930P 3-chip CCD video camera and F100-MPU integrating- frame store, connected to a Nu Vista+ video digitiser in an Apple Macintosh computer.

[0099] GFP-expressing *Arabidopsis* seedlings were removed from agar media, and simply mounted in water under glass coverslips for microscopy. Growing roots could also be directly viewed through coverslip based vessels. Specimens were examined using a BioRad MRC-600 laser-scanning confocal microscope equipped with a 25m W krypton-argon or argon ion laser and filter sets suitable for the detection of fluorescein and texas red dyes (BioRad filter blocks K1/K2 with krypton-argon ion laser, and A1/A2 with argon ion laser). We routinely use a Nikon 60x PlanApo N.A. 1.2 water immersion objective to minimise loss of signal through spherical aberration at long working distances. For the collection of timelapse images, the laser light source was attenuated by 99% using a neutral density filter, the confocal aperture was stopped down and single scans were collected at two second intervals. The large data files were transferred to an Apple Macintosh computer, and the programs PicMerge and 4DTurnaround were used with Adobe Photoshop and Premiere to produce QuickTime movies for display and analysis.

[0100] GFP fluorescence can be seen from 4 days after Agrobacterium inoculation, depending on the expression pattern. The plantlets exhibiting fluorescence can be used to construct a library of transformed plants.

Example 7 Transactivation

[0101] mHAPl-VP16 expression within these lines can be used to direct the expression of a chosen gene at a precise time and place within the organism. The inventors have produced transgenic plants which maintain regulatory proteins or toxins, silent behind a HAP1-responsive promoter. These genes can now be activated in specific cells by crossing to a chosen mHAP1-VP16 expressing line.

[0102] A stable transformed line HJR1 of Arabidopsis thaliana, which forms part of the library described above, expresses modified GFP (under the influence of the mHAP1-VP16 activator) in the cells of the extreme root tip. Similar lines have also been produced which carry a localised cyan fluorescent protein, driven by the mHAP1-VP16 gene.

[0103] Using standard techniques, the line is crossed with another Arabidopsis line which comprises a silently maintained GUS reporter gene under operable control of a HAP1-responsive UAS. The plantlets obtained from the cross

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express GUS under the influence of the HAP1-VP16 transcriptional activator. The pattern of expression is the same as that for the GFP reporter gene in the parent cell line (i.e. at the extreme root tip). Thus the modified HAP1 DNA binding domain sequence is enables the expression of chosen genes of interest (e.g. GUS) in a predictable pattern and enables simultaneous expression of a plurality of genes of interest (e.g. GFP and GUS).

SEQUENCE LISTING

[0104]

<110> Cambridge University Technical Services Limited
Haseloff, Jim
Bauch, Marion
Boisnard-Lorig, Corinne
Hodge, Sarah
Laplaze, Laurent
Runions, John
Kurup, Smita

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Leu Ser Cys Thr Ile Cys Arg Lys Arg Lys Val Lys Cys Asp Lys Leu
            20                  25                  30
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          35                  40                  45

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His Tyr Met Glu Gln Thr Trp Ala Glu Glu Ala Glu Lys Glu Leu Leu
      50                  55                  60

aag gac aac gag ttg aag aag ctc agg gag cgc gtg aag tcc ttg gag   240
Lys Asp Asn Glu Leu Lys Lys Leu Arg Glu Arg Val Lys Ser Leu Glu
 65                  70                  75                  80

aag acc ctc tcc aag gtg cac tcc tcc ccg tcg tcc aac tcc acg gcc   288
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                 85                  90                  95

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Asp Val Ala Met Ala His Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp
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        35                  40                  45

His Tyr Met Glu Gln Thr Trp Ala Glu Glu Ala Glu Lys Glu Leu Leu
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Lys Asp Asn Glu Leu Lys Lys Leu Arg Glu Arg Val Lys Ser Leu Glu
65                  70                  75                  80

Lys Thr Leu Ser Lys Val His Ser Ser Pro Ser Ser Asn Ser Thr Ala
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            100                 105                 110

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Leu Leu Val Val Leu Val Ser Leu Asn Leu Ala Ser Glu Thr Thr Ala
             20                  25                  30

aaa tac act tac tcc tct cca cca cct ccc gag cat tct cct cca ccg  144
Lys Tyr Thr Tyr Ser Ser Pro Pro Pro Pro Glu His Ser Pro Pro Pro
         35                  40                  45

ccg gag cat tct cct cct ccg cct tac cac tac gaa tcc ccg ccc ccg  192
Pro Glu His Ser Pro Pro Pro Pro Tyr His Tyr Glu Ser Pro Pro Pro
     50                  55                  60
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Pro Lys His Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Tyr Lys Ser
 65                  70                  75                  80

cca ccg cct cct atg cat tct cct cca ccg cct tat cat ttt gag tct   288
Pro Pro Pro Pro Met His Ser Pro Pro Pro Pro Tyr His Phe Glu Ser
                85                  90                  95

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Pro Pro Pro Pro Lys His Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys
            100                 105                 110

tac aaa tct cca cca cca cct aaa cat tct cct gca cca gtg cat cat   384
Tyr Lys Ser Pro Pro Pro Pro Lys His Ser Pro Ala Pro Val His His
        115                 120                 125

tat aaa tac aag tct cca cca cca cca aca ccg gtt tat aag tat aaa   432
Tyr Lys Tyr Lys Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Tyr Lys
    130                 135                 140

tct cca cca cca cca aag cat tct cct gca cca gaa cat cac tat aag   480
Ser Pro Pro Pro Pro Lys His Ser Pro Ala Pro Glu His His Tyr Lys
145                 150                 155                 160

tac aag tct cca cca cca cct aag cat ttt cct gca cca gaa cat cac   528
Tyr Lys Ser Pro Pro Pro Pro Lys His Phe Pro Ala Pro Glu His His
                165                 170                 175

tat aag tac aag tac aag tct cca cca cca cca aca ccg gtc tac aag   576
Tyr Lys Tyr Lys Tyr Lys Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys
            180                 185                 190

tat aaa tct cca cca cct cca aca ccg gtc tac aag tac aag tct cca   624
Tyr Lys Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Tyr Lys Ser Pro
        195                 200                 205
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```
cca cca ccc aag cat tct ccc gca cca gta cac cat tac aag tac aag   672
Pro Pro Pro Lys His Ser Pro Ala Pro Val His His Tyr Lys Tyr Lys
    210                 215                 220

tct cca cca cca cca act cca gtt tat aaa tct cca cca cca ccc gaa   720
Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Ser Pro Pro Pro Pro Glu
225                 230                 235                 240

cat tcc cca cca cca cca aca ccg gtc tac aaa tac aag tct cca cca   768
His Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Tyr Lys Ser Pro Pro
                245                 250                 255

cca cca atg cac tct cca cca cca cca aca cca gtt tac aag tac aag   816
Pro Pro Met His Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Tyr Lys
            260                 265                 270

tct ccg cca cca cca atg cac tct ccc cca cca cca gtt tac tct cca   864
Ser Pro Pro Pro Pro Met His Ser Pro Pro Pro Pro Val Tyr Ser Pro
        275                 280                 285

cca cca ccc aaa cat cac tac tcc tat acg tca cct cct cct cct cac   912
Pro Pro Pro Lys His His Tyr Ser Tyr Thr Ser Pro Pro Pro Pro His
    290                 295                 300

cac tac gaa ttc gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg   960
His Tyr Glu Phe Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val
305                 310                 315                 320

ccc atc ctg gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc   1008
Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser
                325                 330                 335

gtg tcc ggc gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg   1056
Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu
            340                 345                 350
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```
aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc   1104
Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu
        355                 360                 365

gtg acc acc ttg acc tac ggc gtg cag tgc ttc gcc cgc tac ccc gac   1152
Val Thr Thr Leu Thr Tyr Gly Val Gln Cys Phe Ala Arg Tyr Pro Asp
    370                 375                 380

cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac   1200
His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr
385                 390                 395                 400

gtc cag gag cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc   1248
Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr
                405                 410                 415

cgc gcc gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag   1296
Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu
            420                 425                 430

ctg aag ggc atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag   1344
Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys
        435                 440                 445

ctg gag tac aac tac aac agc cac aag gtc tat atc acc gcc gac aag   1392
Leu Glu Tyr Asn Tyr Asn Ser His Lys Val Tyr Ile Thr Ala Asp Lys
    450                 455                 460

cag aag aac ggc atc aag gtg aac ttc aag acc cgc cac aac atc gag   1440
Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu
465                 470                 475                 480

gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc   1488
Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile
                485                 490                 495
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```
ggc gac ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag   1536
Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln
            500                 505                 510

tcc gcc ctg agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg   1584
Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu
        515                 520                 525

ctg gag ttc gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg   1632
Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu
    530                 535                 540

tac aag taa                                                       1641
Tyr Lys
545
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<210> 14
<211> 546
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Encoded amino acid sequence of extensin-GFP fusion

<400> 14

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Met Gly Arg Ile Ala Arg Gly Ser Lys Met Ser Ser Leu Ile Val Ser
  1               5                  10                  15

Leu Leu Val Val Leu Val Ser Leu Asn Leu Ala Ser Glu Thr Thr Ala
            20                  25                  30

Lys Tyr Thr Tyr Ser Ser Pro Pro Pro Pro Glu His Ser Pro Pro Pro
        35                  40                  45

Pro Glu His Ser Pro Pro Pro Pro Tyr His Tyr Glu Ser Pro Pro Pro
    50                  55                  60
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Pro Lys His Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Tyr Lys Ser
65 70 75 80

Pro Pro Pro Pro Met His Ser Pro Pro Pro Pro Tyr His Phe Glu Ser
85 90 95

Pro Pro Pro Pro Lys His Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys
100 105 110

Tyr Lys Ser Pro Pro Pro Pro Lys His Ser Pro Ala Pro Val His His
115 120 125

Tyr Lys Tyr Lys Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Tyr Lys
130 135 140

Ser Pro Pro Pro Pro Lys His Ser Pro Ala Pro Glu His His Tyr Lys
145 150 155 160

Tyr Lys Ser Pro Pro Pro Pro Lys His Phe Pro Ala Pro Glu His His
165 170 175

Tyr Lys Tyr Lys Tyr Lys Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys
180 185 190

Tyr Lys Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Tyr Lys Ser Pro
195 200 205

Pro Pro Pro Lys His Ser Pro Ala Pro Val His His Tyr Lys Tyr Lys
210 215 220

Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Ser Pro Pro Pro Pro Glu
225 230 235 240

His Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Tyr Lys Ser Pro Pro
245 250 255

Pro Pro Met His Ser Pro Pro Pro Pro Thr Pro Val Tyr Lys Tyr Lys
260 265 270

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Ser Pro Pro Pro Pro Met His Ser Pro Pro Pro Pro Val Tyr Ser Pro
275 280 285

Pro Pro Pro Lys His His Tyr Ser Tyr Thr Ser Pro Pro Pro Pro His
290 295 300

His Tyr Glu Phe Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val
305 310 315 320

Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser
325 330 335

Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu
340 345 350

Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu
355 360 365

Val Thr Thr Leu Thr Tyr Gly Val Gln Cys Phe Ala Arg Tyr Pro Asp
370 375 380

His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr
385 390 395 400

Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr
405 410 415

Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu
420 425 430

Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys
435 440 445

Leu Glu Tyr Asn Tyr Asn Ser His Lys Val Tyr Ile Thr Ala Asp Lys
450 455 460

Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu
465 470 475 480

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Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile
485 490 495

Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln
500 505 510

Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu
515 520 525

Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu
530 535 540

Tyr Lys
545

<210> 15
<211> 106
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:

oligonucleotide used in construction of a HAP1 DNA binding site

<400> 15

agcttagcac ggacttatcg gtcggagcac ggacttatcg gtcggagcac ggacttatcg 60
gtcggagcac ggacttatcg gtcggagcac ggacttatcg gtcggt 106

<210> 16
<211> 106
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:

Oligonucleotide used in construction of a HAP1 DNA binding site

<400> 16

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ctagaccgac cgataagtcc gtgctccgac cgataagtcc gtgctccgac cgataagtcc 60
gtgctccgac cgataagtcc gtgctccgac cgataagtcc gtgcta 106

<210> 17
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 17
ggcggatcca acaatgggaa gaattgctag aggctc 36

<210> 18
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 18
ggcggattcg tagtggtgag gaggaggagg tgacgt 36

Claims

1. An isolated nucleic acid molecule comprising a modified HAP1 DNA-binding domain nucleotide sequence encoding at least an effective portion of a HAP1 DNA-binding domain fused to a second nucleotide sequence which encodes a transcriptional activator domain such as to encode a HAP1 transcriptional activator,
characterised in that said modified nucleotide sequence has an A/T base content substantially reduced compared to the wild-type sequence such as to be expressible in a plant cell, wherein the modified nucleotide sequence has the sequence of Figure 2b (middle line) (SEQ. ID. NO. 10).

2. A nucleic acid molecule as claimed in claim 1 wherein the transcriptional activator domain is selected from the activation domain of the HAP1 protein or herpes simplex virus (HSV) VP-16.

3. A nucleic acid molecule as claimed in claim 2 wherein the modified nucleotide sequence and the second nucleotide sequence encode the amino acid sequence of the mHAP1-VPI6 chimera shown in Figure 2c (bottom line) (SEQ.ID.NO.12).

4. A nucleic acid molecule as claimed in claim 3 wherein the modified nucleotide sequence and second nucleotide sequence consist of the sequence shown in Figure 2c (top line) (SEQ. ID. No. 11).

5. A vector which comprises the nucleic acid comprising a modified HAP1 DNA-binding domain nucleotide sequence encoding at least an effective portion of a HAP1 DNA-binding domain fused to a second nucleotide sequence which encodes a transcriptional activator domain such as to encode a HAP1 transcriptional activator, wherein said modified nucleotide sequence has an A/T base content of less than 45%
wherein the nucleic acid is operably linked to a promoter for transcription in a host plant cell,
and wherein the promoter is an enhancer dependent promoter such that if the promoter is inserted into a plant host cell genome in functional relationship with an enhancer sequence and required transcription factors, the promoter will direct expression in a tissue specific manner,
which vector further comprises a heterologous nucleotide sequence consisting of a heterologous gene operably linked to a HAP1 upstream activation sequence.

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6. A composition of matter comprising a pair of vectors, which vectors are:

(i) a vector which comprises the nucleic acid comprising a modified HAP1 DNA-binding domain nucleotide sequence encoding at least an effective portion of a HAP1 DNA-binding domain fused to a second nucleotide sequence which encodes a transcriptional activator domain such as to encode a HAP1 transcriptional activator, wherein said modified nucleotide sequence has an A/T base content of less than 45%
wherein the nucleic acid is operably linked to a promoter for transcription in a host plant cell,
and wherein the promoter is an enhancer dependent promoter such that if the promoter is inserted into a plant host cell genome in functional relationship with an enhancer sequence and required transcription factors, the promoter will direct expression in a tissue specific manner,
(ii) a vector comprising a nucleic acid which includes a heterologous nucleotide sequence consisting of a heterologous gene operably linked to a HAP1 upstream activation sequence.

7. A vector or pair of vectors as claimed in claim 5 or claim 6, wherein the effective portion comprises amino acid residues 1 to 94 of the yeast HAP1 polypeptide.

8. A vector or pair of vectors as claimed in any one of claims 5 to 7, wherein the % A/T base content of the modified nucleotide sequence is less than 45%.

9. A vector or pair of vectors as claimed in claim 8, wherein the effective portion of a HAP1 DNA-binding domain has the amino acid sequence of Figure 2b (bottom line) (SEQ. ID. NO.9).

10. A vector or pair of vectors as claimed in claim 9, wherein the nucleic acid is as claimed in any one of claims 1 to 4.

11. A vector or pair of vectors as claimed in any one of claims 5 to 7 comprising right and left Ti-DNA, to enable stable insertion into the genome of a plant host cell.

12. A vector or pair of vectors as claimed in any one of claims 5 to 11, wherein the heterologous gene is a reporter gene.

13. A vector or pair of vectors as claimed in claim 12 wherein the reporter gene encodes a reporter polypeptide capable of generating a visually detectable signal.

14. A vector or pair of vectors as claimed in claim 13 wherein the visually detectable signal can be monitored by multispectral dynamic imaging.

15. A vector or pair of vectors as claimed in claim 13 or claim 14 wherein the reporter polypeptide is a wild-type or modified GFP.

16. A vector or pair of vectors as claimed in claim 15 wherein the modified GFP is a GFP extensin reporter gene fusion.

17. A vector or pair of vectors as claimed in claim 16 wherein the GFP extensin reporter gene fusion is a green fluorescent protein linked to an effective portion of extensin.

18. A vector or pair of vectors as claimed in claim 17 wherein the effective portion comprises at least 60% of the full-length sequence of a carrot extensin polypeptide.

19. A vector or pair of vectors as claimed in claim 18 wherein the reporter gene nucleotide sequence encodes the amino acid sequence of Figure 3b (bottom line) (SEQ. ID. NO. 14).

20. A vector or pair of vectors as claimed in claim 19 wherein the reporter gene nucleotide sequence has the sequence of Figure 3a (top line).

21. A vector or pair of vectors as claimed in any one of claims 5 to 20 wherein the enhancer dependent promoter comprises a TATA box.

22. A method which comprises the step of introducing the vector or pair of vectors of any one of claims 5 to 21 into a host plant cell, and optionally causing or allowing recombination between the vector or vectors and the plant cell genome such as to transform the host cell.

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23. A plant cell containing or transformed with the vector or pair of vectors of any one of claims 5 to 21.

24. A method for producing a transgenic plant, which method comprises the steps of:

(a) performing a method as claimed in claim 22,
(b) regenerating a plant from the transformed plant cell.

25. A transgenic plant which is obtainable by the method of claim 24, or which is a clone, or selfed or hybrid progeny or other descendant of said transgenic plant, which in each case includes the nucleic acid comprising the modified HAP1 DNA-binding domain described in any one of claims 1 to 21.

26. A plant as claimed in claim 25 which has integrated therein a reporter nucleotide sequence consisting of a reporter gene operably linked to a HAP1 upstream activation sequence which reporter nucleotide sequence is as described in any one of claims 12 to 20.

27. A population of plants as claimed in claim 26 wherein the integrated reporter gene differs between said plants, plantlets or parts thereof.

28. A method comprising introducing a gene of interest into the plant or plants of claim 26 or claim 27 which gene of interest is operable linked to a HAP1 upstream activation sequence,
such that the pattern of expression of the gene of interest is the same as that of the reporter gene.

29. A method as claimed in claim 28 wherein the gene of interest is introduced and thereby trans-activated by crossing.

30. A method as claimed in claim 28 wherein the gene of interest is introduced by means of a plant vector.

31. A method of identifying a plant enhancer nucleic acid sequence, comprising the steps of:

(i) transforming a plant cell host with a vector as claimed in claim 13,
(ii) observing said plant expression of said HAP1 binding domain, and
(iii) optionally, characterising the position and\or nucleic acid sequence of the enhancer sequence.

32. A method of controlling expression of a gene of interest in a plant, the method comprising the steps of:

(i) providing a plant comprising a nucleic acid encoding a HAP1 transcriptional activator as defined in any one of claims 1 to 4 under the control of a naive promoter such that expression of the HAP1 transcriptional activator is limited to those cell types in which a naive promoter sequence is in functional relationship with a host cell enhancer sequence and required transcription factors,
(ii) introducing the gene of interest into said plant or part thereof, said gene of interest having an HAP1 responsive upstream activation sequence,

wherein binding of said HAP1 transcriptional activator to said upstream activator sequence causes transcriptional activation of the gene of interest.

33. A method as claimed in claim 32 wherein the gene of interest encodes a toxin.

34. A method as claimed in claim 32 wherein the gene of interest is of unknown function.

35. A method of determining the function of a gene of interest comprising the steps of:

(i) performing a method as claimed in claim 34,
(ii) comparing the phenotype of said plant or part thereof in which said gene of interest is expressed with a second plant or part thereof in which said gene of interest is not expressed.

36. A method of independently controlling expression of a first and a second gene of interest in a plant comprising the steps of:

(i) providing a plant comprising a nucleic acid encoding a HAP1 transcriptional activator as defined in any one

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of claims 1 to 4, and a second nucleic acid sequence, which encodes a GAL4 transcriptional activator;
(ii) introducing the first gene of interest into a plant or part thereof, said first gene of interest having an HAP1 responsive upstream activation sequence; introducing the second gene of interest into a plant or part thereof, said second gene of interest having a GAL4 responsive upstream activation sequence;

wherein binding of said HAP1 transcriptional activator to said upstream activator sequence causes transcriptional activation of the first gene of interest and binding of said GAL4 transcriptional activator to said upstream activator sequence causes transcriptional activation of the second gene of interest.

37. A method of co-ordinating the expression of a plurality of genes of interest in a plant or part thereof, comprising the steps of:

(i) providing a plant comprising a nucleic acid encoding a HAP1 transcriptional activator as defined in any one of claims 1 to 4, and a second nucleic acid sequence, which encodes a GAL4 transcriptional activator;
(ii) introducing the genes of interest into a plant or part thereof, said genes of interest each being under the control of an HAP1 responsive upstream activation sequence,

wherein binding of said HAP1 transcriptional activator to said upstream activator sequence causes transcriptional activation of the genes of interest.

38. A method as claimed in claim 37 wherein the plurality of genes are associated with a single upstream activator sequence.

Patentansprüche

1. Isoliertes Nucleinsäuremolekül, umfassend eine modifizierte HAP1-DNA-Bindungsdomänen-Nucleotidsequenz, die für zumindest einen wirksamen Abschnitt einer HAP1-DNA-Bindungsdomäne kodiert, fusioniert an eine zweite Nucleotidsequenz, die für eine Transkriptionsaktivatordomäne kodiert, um für einen HAP1-Transkriptionsaktivator zu kodieren,
dadurch gekennzeichnet, dass die modifizierte Nucleotidsequenz einen im Vergleich zur Wildtypsequenz wesentlich reduzierten A/T-Basengehalt aufweist, so dass sie in einer Pflanzenzelle exprimierbar ist, worin die modifizierte Nucleotidsequenz die Sequenz aus Fig. 2b (mittlere Reihe) (Seq.-ID Nr. 10) aufweist.

2. Nucleinsäuremolekül nach Anspruch 1, worin die Transkriptionsaktivatordomäne aus der Aktivierungsdomäne des HAP1-Proteins oder des Herpes-Simplex-Virus-(HSV-) VP-16 ausgewählt ist.

3. Nucleinsäuremolekül nach Anspruch 2, worin die modifizierte Nucleotidsequenz und die zweite Nucleotidsequenz für die Aminosäuresequenz der in Fig. 2c (untere Reihe) gezeigten mHAP1-VP16-Chimäre (Seq.-ID Nr. 12) kodieren.

4. Nucleinsäuremolekül nach Anspruch 3, worin die modifizierte Nucleotidsequenz und die zweite Nucleotidsequenz aus der in Fig. 2c (obere Reihe) gezeigten Sequenz (Seq.-ID Nr. 11) bestehen.

5. Vektor, der Nucleinsäure umfasst, die eine modifizierte HAP1-DNA-Bindungsdomänen-Nucleotidsequenz, die zumindest für einen wirksamen Abschnitt einer HAP1-DNA-Bindungsdomäne kodiert, fusioniert an eine zweite Nucleotidsequenz, umfasst, die für eine Transkriptionsaktivatordomäne kodiert, um für einen HAP1-Transkriptionsaktivator zu kodieren, worin die modifizierte Nucleotidsequenz einen A/T-Basengehalt von weniger als 45 % aufweist, worin die Nucleinsäure operabel an einen Promotor für die Transkription in einer Wirts-Pflanzenzelle gebunden ist, und worin der Promotor ein Enhancer-abhängiger Promotor ist, so dass, sofern der Promotor in funktioneller Beziehung mit einer Enhancer-Sequenz und erforderlichen Transkriptionsfaktoren in ein Pflanzen-Wirtszellgenom insertiert wird, der Promotor die Expression gewebespezifisch steuert,
worin der Vektor weiters eine heterologe Nucleotidsequenz umfasst, die aus einem heterologen Gen, operabel an eine HAP1-Stromauf-Aktivierungssequenz gebunden, besteht.

6. Materialzusammensetzung, umfassend ein Vektorenpaar, worin die Vektoren folgende sind:

(i) ein Vektor, der Nucleinsäure umfasst, die eine modifizierte HAP1-DNA-Bindungsdomänen-Nucleotidsequenz, die für zumindest einen wirksamen Abschnitt einer HAP1-DNA-Bindungsdomäne kodiert, fusioniert an

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eine zweite Nucleotidsequenz, umfasst, die für eine Transkriptionsaktivatordomäne kodiert, um für einen HAP1-Transkriptionsaktivator zu kodieren, worin die modifizierte Nucleotidsequenz einen A/T-Basengehalt von weniger als 45 % aufweist,
worin die Nucleinsäure operabel an einen Promotor für die Transkription in einer Wirts-Pflanzenzelle gebunden ist,
und worin der Promotor ein Enhancer-abhängiger Promotor ist, so dass, sofern der Promotor in funktioneller Beziehung mit einer Enhancer-Sequenz und erforderlichen Transkriptionsfaktoren in ein Pflanzen-Wirtszellgenom insertiert wird, der Promotor die Expression gewebespezifisch steuert,
(ii) ein Vektor, der Nucleinsäure umfasst, die eine heterologe Nucleotidsequenz enthält, die aus einem heterologen Gen, operabel an eine HAP1-Stromauf-Aktivierungssequenz gebunden, besteht.

7. Vektor oder Vektorenpaar nach Anspruch 5 oder Anspruch 6, worin der wirksame Abschnitt die Aminosäurereste 1 bis 94 des Hefe-HAP1-Polypeptids umfasst.

8. Vektor oder Vektorenpaar nach Anspruch 5 bis Anspruch 7, worin der prozentuelle A/T-Basengehalt der modifizierten Nucleotidsequenz weniger als 45 % beträgt.

9. Vektor oder Vektorenpaar nach Anspruch 8, worin der wirksame Abschnitt einer HAP1-DNA-Bindungsdomäne die Aminosäuresequenz aus Fig. 2b (untere Reihe) (Seq.-ID Nr. 9) aufweist.

10. Vektor oder Vektorenpaar nach Anspruch 9, worin die Nucleinsäure wie in einem der Ansprüche 1 bis 4 definiert ist.

11. Vektor oder Vektorenpaar nach einem der Ansprüche 5 bis 7, umfassend rechte und linke Ti-DNA, um stabile Insertion in das Genom einer Pflanzenwirtszelle zu ermöglichen.

12. Vektor oder Vektorenpaar nach einem der Ansprüche 5 bis 11, worin das heterologe Gen ein Reportergen ist.

13. Vektor oder Vektorenpaar nach Anspruch 12, worin das Reportergen für ein Reporterpolypeptid kodiert, das ein visuell nachweisbares Signal erzeugen kann.

14. Vektor oder Vektorenpaar nach Anspruch 13, worin das visuell nachweisbare Signal durch multispektrale dynamische Bildgebung verfolgt werden kann.

15. Vektor oder Vektorenpaar nach Anspruch 13 oder Anspruch 14, worin das Reporterpolypeptid ein Wildtyp- oder modifiziertes GFP ist.

16. Vektor oder Vektorenpaar nach Anspruch 15, worin das modifizierte GFP eine GFP-Extensin-Reportergen-Fusion ist.

17. Vektor oder Vektorenpaar nach Anspruch 16, worin die GFP-Extensin-Reportergen-Fusion ein grün fluoreszierendes Protein, gebunden an einen wirksamen Abschnitt von Extensin, ist.

18. Vektor oder Vektorenpaar nach Anspruch 17, worin der wirksame Abschnitt zumindest 60 % der Volllängensequenz eines Karotten-Extensin-Polypeptids umfasst.

19. Vektor oder Vektorenpaar nach Anspruch 18, worin die Reportergen-Nucleotidsequenz für die Aminosäuresequenz aus Fig. 3b (untere Reihe) (Seq.-ID Nr. 14) kodiert.

20. Vektor oder Vektorenpaar nach Anspruch 19, worin die Reportergen-Nucleotidsequenz die Sequenz aus Fig. 3a (obere Reihe) aufweist.

21. Vektor oder Vektorenpaar nach einem der Ansprüche 5 bis 20, worin der Enhancer-abhängige Promotor eine TATA-Box umfasst.

22. Verfahren, das den Schritt des Einführens eines Vektors oder Vektorenpaars nach einem der Ansprüche 5 bis 21 in eine Wirtspflanzenzelle und gegebenenfalls des Auslösens oder Ermöglichens von Rekombination zwischen dem Vektor oder den Vektoren und dem Pflanzenzellgenom, um die Wirtszelle zu transformieren, umfasst.

23. Pflanzenzelle, die einen Vektor oder ein Vektorenpaar nach einem der Ansprüche 5 bis 21 enthält oder damit

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transformiert ist.

24. Verfahren zur Herstellung einer transgenen Pflanze, die folgenden Schritte umfassend:

(a) Durchführung eines Verfahrens nach Anspruch 22,
(b) Neubildung einer Pflanze aus der transformierten Pflanzenzelle.

25. Transgene Pflanze, die durch ein Verfahren nach Anspruch 24 erhältlich ist oder die ein Klon oder eine geselbstete oder Hybrid-Nachkommenschaft oder ein anderer Abkömmling der transgenen Pflanze ist, die bzw. der in jedem Fall Nucleinsäure aufweist, welche die in einem der Ansprüche 1 bis 21 beschriebene modifizierte HAP1-DNA-Bindungsdomäne umfasst.

26. Pflanze nach Anspruch 25, die eine integrierte Reporter-Nucleotidsequenz aufweist, die aus einem an eine HAP1-Stromauf-Aktivierungssequenz operabel gebundenen Reportergen besteht, worin die Reporter-Nucleotidsequenz wie in den Ansprüchen 12 bis 20 beschrieben ist.

27. Population von Pflanzen nach Anspruch 26, worin das integrierte Reportergen zwischen den Pflanzen, Jungpflanzen und Teilen davon unterscheidet.

28. Verfahren, umfassend das Einführen eines Gens von Interesse in eine Pflanze oder Pflanzen nach Anspruch 26 oder Anspruch 27,
worin das Gen von Interesse operabel an eine HAP1-Stromauf-Aktivierungssequenz gebunden ist,
so dass das Expressionsmuster des Gens von Interesse dasselbe wie jenes des Reportergens ist.

29. Verfahren nach Anspruch 28, worin das Gen von Interesse eingeführt und **dadurch** durch Kreuzung transaktiviert wird.

30. Verfahren nach Anspruch 28, worin das Gen von Interesse mittels eines Pflanzenvektors eingeführt wird.

31. Verfahren zur Identifikation einer Pflanzen-Enhancer-Nucleinsäuresequenz, die folgenden Schritte umfassend:

(i) Transformieren eines Pflanzenzellwirts mit einem Vektor nach Anspruch 13,
(ii) Beobachten der Pflanzenexpression der HAP1-Bindungsdomäne und
(iii) gegebenenfalls Charakterisieren der Position und/oder der Nucleinsäuresequenz der Enhancer-Sequenz.

32. Verfahren zur Steuerung von Expression eines Gens von Interesse in einer Pflanze, die folgenden Schritte umfassend:

(i) Bereitstellen einer Pflanze, die Nucleinsäure umfasst, die für einen in einem der Ansprüche 1 bis 4 definierten HAP1-Transkriptionsaktivator kodiert, unter der Steuerung eines naiven Promotors, so dass Expression des HAP1-Transkriptionsaktivators auf jene Zelltypen beschränkt wird, in denen eine naive Promotorsequenz in funktioneller Beziehung mit einer Wirtszellen-Enhancer-Sequenz und erforderlichen Transkriptionsfaktoren steht,
(ii) das Einführen des Gens von Interesse in die Pflanze oder einen Teil davon, worin das Gen von Interesse eine auf HAP1 ansprechende Stromauf-Aktivierungssequenz aufweist,

worin Bindung des HAP1-Transkriptionsaktivators an die Stromauf-Aktivatorsequenz Transkriptionsaktivierung des Gens von Interesse bewirkt.

33. Verfahren nach Anspruch 32, worin das Gen von Interesse für ein Toxin kodiert.

34. Verfahren nach Anspruch 32, worin das Gen von Interesse eine unbekannte Funktion hat.

35. Verfahren zur Bestimmung der Funktion eines Gens von Interesse, die folgenden Schritte umfassend:

(i) Durchführung eines Verfahrens nach Anspruch 34,
(ii) Vergleichen des Phänotyps der Pflanze oder des Teils davon, in der/dem das Gen von Interesse exprimiert wird, mit einer zweiten Pflanze oder einem Teil davon, in der/dem das Gen von Interesse nicht exprimiert wird.

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36. Verfahren zur unabhängigen Steuerung der Expression eines ersten und eines zweiten Gens von Interesse in einer Pflanze, die folgenden Schritte umfassend:

(i) Bereitstellung einer Pflanze, die Nucleinsäure, die für einen HAP1-Transkriptionsaktivator kodiert, wie in einem der Ansprüche 1 bis 4 definiert, und eine zweite Nucleinsäuresequenz umfasst, die für einen GAL4-Transkriptionsaktivator kodiert;
(ii) Einführung des ersten Gens von Interesse in eine Pflanze oder einen Teil davon, worin das erste Gen von Interesse eine auf HAP1 ansprechende Stromauf-Aktivierungssequenz aufweist;

Einführung des zweiten Gens von Interesse in eine Pflanze oder einen Teil davon, worin das zweite Gen von Interesse eine auf GAL4 ansprechende Stromauf-Aktivierungssequenz aufweist;
worin Bindung des HAP1-Transkriptionsaktivators an die Stromauf-Aktivatorsequenz Transkriptionsaktivierung des erstens Gens von Interesse bewirkt und Bindung des GAL4-Transkriptionsaktivators an die Stromauf-Aktivatorsequenz Transkriptionsaktivierung des zweiten Gens von Interesse bewirkt.

37. Verfahren zur Koordinierung der Expression mehrerer Gene von Interesse in einer Pflanze oder einem Teil davon, die folgenden Schritte umfassend:

(i) Bereitstellung einer Pflanze, die Nucleinsäure, die für einen HAP1-Transkriptionsaktivator kodiert, wie in einem der Ansprüche 1 bis 4 definiert, und eine zweite Nucleinsäuresequenz umfasst, die für einen GAL4-Transkriptionsaktivator kodiert;
(ii) Einführung der Gene von Interesse in eine Pflanze oder einen Teil davon, worin die Gene von Interesse unter der Steuerung einer auf HAP1 ansprechenden Stromauf-Aktivierungssequenz stehen;

worin Bindung des HAP1-Transkriptionsaktivators an die Stromauf-Aktivatorsequenz Transkriptionsaktivierung der Gene von Interesse bewirkt.

38. Verfahren nach Anspruch 37, worin die mehreren Gene mit einer einzigen Stromauf-Aktivatorsequenz assoziiert sind.

Revendications

1. Molécule d'acide nucléique isolée comprenant une séquence nucléotidique de domaine de liaison à l'ADN HAP1 modifiée codant au moins une partie efficace d'un domaine de liaison à l'ADN HAP1 fusionnée à une seconde séquence nucléotidique qui code un domaine d'activateur de la transcription de manière à coder un activateur de la transcription d'HAPI;
caractérisée en ce que ladite séquence nucléotidique modifiée a une teneur en base A/T sensiblement réduite comparativement à la séquence sauvage de manière à pouvoir être exprimée dans une cellule végétale, la séquence nucléotidique modifiée ayant la séquence de la figure 2b (ligne du milieu) (SEQ ID N° 10).

2. Molécule d'acide nucléique selon la revendication 1 dans laquelle le domaine d'activateur de la transcription est choisi parmi le domaine d'activation de la protéine HAP1 ou VP-16 du virus herpès simplex (HSV).

3. Molécule d'acide nucléique selon la revendication 1 dans laquelle la séquence nucléotidique modifiée et la seconde séquence nucléotidique codent la séquence d'acides aminés de la chimère mHAP1-VP16 présentée sur la figure 2c (ligne du bas) (SEQ ID N° 12).

4. Molécule d'acide nucléique selon la revendication 3 dans laquelle la séquence nucléotidique modifiée et la seconde séquence nucléotidique consistent en la séquence présentée sur la figure 2c (ligne du haut) (SEQ ID N° 11).

5. Vecteur qui comprend l'acide nucléique comprenant une séquence nucléotidique de domaine de liaison à l'ADN HAP1 codant au moins une partie efficace d'un domaine de liaison à l'ADN HAP1 fusionnée à une seconde séquence nucléotidique qui code un domaine d'activateur de la transcription de manière à coder un activateur de la transcription d'HAP1, dans lequel ladite séquence nucléotidique modifiée a une teneur en base A/T qui n'est pas inférieure à 45 %, dans lequel l'acide nucléique est lié de manière opérationnelle à un promoteur pour transcription dans une cellule végétale hôte,
et dans lequel le promoteur est un promoteur dépendant de l'amplificateur, de sorte que, si le promoteur est inséré dans le génome d'une cellule végétale hôte en relation fonctionnelle avec une séquence d'amplificateur et des

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facteurs de transcription requis, le promoteur dirige l'expression de manière spécifique de tissu,
lequel vecteur comprenant en outre une séquence nucléotidique hétérologue consistant en un gène hétérologue lié de manière opérationnelle à une séquence d'activation amont HAP1.

6. Composition de matière comprenant une paire de vecteurs, lesquels vecteurs étant:

(i) un vecteur qui comprend l'acide nucléique comprenant une séquence nucléotidique de domaine de liaison à l'ADN HAP1 modifiée codant au moins une partie efficace d'un domaine de liaison à l'ADN HAP1 fusionnée à une seconde séquence nucléotidique qui code un domaine d'activateur de la transcription de manière à coder un activateur de la transcription d'HAP1; dans lequel ladite séquence nucléotidique modifiée a une teneur en base A/T qui n'est pas inférieure à 45 %,
dans lequel l'acide nucléique est lié de manière opérationnelle à un promoteur pour transcription dans une cellule végétale hôte,
et dans lequel le promoteur est un promoteur dépendant de l'amplificateur, de sorte que, si le promoteur est inséré dans le génome d'une cellule végétale hôte en relation fonctionnelle avec une séquence d'amplificateur et des facteurs de transcription requis, le promoteur dirige l'expression de manière spécifique de tissu,
(ii) un vecteur comprenant un acide nucléique qui inclut une séquence nucléotidique hétérologue consistant en un gène hétérologue lié de manière opérationnelle à une séquence d'activation amont d'HAP1.

7. Vecteur ou paire de vecteurs selon la revendication 5 ou la revendication 6 dans lesquels la partie efficace comprend les résidus d'acides aminés 1 à 94 du polypeptide HAP1 de levure.

8. Vecteur ou paire de vecteurs selon l'une quelconque des revendications 5 à 7, dans lesquels la teneur en base A/T en pourcentage de la séquence nucléotidique modifiée est inférieur à 45 %.

9. Vecteur ou paire de vecteurs selon la revendication 8, dans lesquels la partie efficace d'un domaine de liaison à l'ADN HAP1 a la séquence d'acides aminés de la figure 2b (ligne du bas) (SEQ ID N° 9).

10. Vecteur ou paire de vecteurs selon la revendication 9, dans lesquels l'acide nucléique est conforme à l'une quelconque des revendications 1 à 4.

11. Vecteur ou paire de vecteurs selon l'une quelconque des revendications 5 à 7 comprenant Ti-DNA droit et gauche pour permettre l'insertion stable dans le génome d'une cellule végétale hôte.

12. Vecteur ou paire de vecteurs selon l'une quelconque des revendications 5 à 11 dans lesquels le gène hétérologue est un gène rapporteur.

13. Vecteur ou paire de vecteurs selon la revendication 12 dans lesquels le gène rapporteur code un polypeptide rapporteur capable de générer un signal détectable visuellement.

14. Vecteur ou paire de vecteurs selon la revendication 13 dans lesquels le signal détectable visuellement peut être surveillé par imagerie dynamique multispectrale.

15. Vecteur ou paire de vecteurs selon la revendication 13 ou la revendication 14 dans lesquels le polypeptide rapporteur est un GFP sauvage ou modifié.

16. Vecteur ou paire de vecteurs selon la revendication 15 dans lesquels le GFP modifié est une fusion GFP extensine gène rapporteur.

17. Vecteur ou paire de vecteurs selon la revendication 16 dans lesquels la fusion GFP extensine gène rapporteur est une protéine fluorescente liée à une partie efficace de l'extensine.

18. Vecteur ou paire de vecteurs selon la revendication 17 dans lesquels la partie efficace comprend au moins 60 % de la séquence complète d'un polypeptide d'extensine de carotte.

19. Vecteur ou paire de vecteurs selon la revendication 18 dans lesquels la séquence nucléotidique de gène rapporteur code la séquence d'acides aminés de la figure 3b (ligne du bas) (SEQ ID N° 14).

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20. Vecteur ou paire de vecteurs selon la revendication 19 dans lesquels la séquence nucléotidique de gène rapporteur a la séquence de la figure 3a (ligne du haut).

21. Vecteur ou paire de vecteurs selon l'une quelconque des revendications 5 à 20 dans lesquels le promoteur dépendant de l'amplificateur comprend une boîte TATA.

22. Procédé qui comprend l'étape consistant à introduire le vecteur ou la paire de vecteurs selon l'une quelconque des revendications 5 à 21 dans une cellule végétale hôte, et facultativement à provoquer ou à permettre une recombinaison entre le ou les vecteurs et le génome de la cellule végétale, de manière à transformer la cellule hôte.

23. Cellule végétale contenant le vecteur ou la paire de vecteurs selon l'une quelconque des revendications 5 à 21 ou transformée avec ce vecteur ou cette paire de vecteurs.

24. Procédé pour produire une plante transgénique, lequel procédé comprenant les étapes consistant à:

(a) réaliser un procédé selon la revendication 22;
(b) régénérer une plante à partir de la cellule végétale transformée.

25. Plante transgénique que l'on peut obtenir par le procédé de la revendication 24, ou qui est un clone ou une progéniture produite par autofécondation ou une progéniture hybride ou une autre descendance de ladite plante transgénique, qui dans chaque cas inclut l'acide nucléique comprenant le domaine de liaison à l'ADN HAP1 modifié décrit dans l'une quelconque des revendications 1 à 21.

26. Plante selon la revendication 25 dans laquelle est intégrée une séquence nucléotidique de rapporteur consistant en un gène de rapporteur lié de manière opérationnelle à une séquence d'activation amont d'HAP1, laquelle séquence nucléotidique de rapporteur est comme décrite dans l'une quelconque des revendications 12 à 20.

27. Population de plantes selon la revendication 26 dans laquelle le gène de rapporteur intégré diffère entre lesdites plantes, plantules ou parties de celles-ci.

28. Procédé comprenant l'introduction d'un gène d'intérêt dans la ou les plantes selon la revendication 26 ou la revendication 27
lequel gène d'intérêt étant lié de manière opérationnelle à une séquence d'activation amont d'HAP1,
de sorte que le motif d'expression du gène d'intérêt est le même que celui du gène de rapporteur.

29. Procédé selon la revendication 28 dans lequel le gène d'intérêt est introduit et ainsi transactivé par croisement.

30. Procédé selon la revendication 28 dans lequel le gène d'intérêt est introduit en utilisant un vecteur végétal.

31. Procédé d'identification d'une séquence d'acide nucléique d'amplificateur végétal comprenant les étapes consistant à:

(i) transformer une cellule végétale hôte avec un vecteur selon la revendication 13,
(ii) observer ladite expression végétale dudit domaine de liaison d'HAP1, et
(iii) facultativement caractériser la position et/ou la séquence d'acides nucléique de la séquence d'amplificateur.

32. Procédé pour contrôler l'expression d'un gène d'intérêt dans un e plante, le procédé comprenant les étapes consistant à:

(i) fournir une plante comprenant un acide nucléique codant un activateur de la transcription d'HAP1 comme défini dans l'une quelconque des revendications 1 à 4 sous le contrôle d'un promoteur naïf de sorte que l'expression de l'activateur de la transcription d'HAP1 est limitée aux types de cellules dans lesquelles une séquence de promoteur naïf est en relation fonctionnelle avec une séquence d'amplificateur de cellule hôte et des facteurs de transcription requis,
(ii) introduire le gène d'intérêt dans ladite plante ou une partie de celle -ci, ledit gène d'intérêt ayant une séquence d'activation amont répondant à HAP1,

dans lequel la liaison dudit activateur de la transcription d'HAP1 à ladite séquence d'activateur amont provoque

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l'activation de la transcription du gène d'intérêt.

33. Procédé selon la revendication 32 dans lequel le gène d'intérêt code une toxine.

34. Procédé selon la revendication 32 dans lequel le gène d'intérêt remplit une fonction inconnue.

35. Procédé de détermination de la fonction d'un gène d'intérêt comprenant les étapes consistant à:

(i) réaliser un procédé selon la revendication 34;
(ii) comparer le phénotype de ladite plante ou d'une partie de celle-ci dans laquelle ledit gène d'intérêt est exprimé avec une seconde plante ou une partie de celle-ci dans laquelle ledit gène d'intérêt n'est pas exprimé.

36. Procédé pour contrôler indépendamment l'expression d'un premier gène et d'un second gène d'intérêt dans une plante comprenant les étapes consistant à:

(i) fournir une plante comprenant un acide nucléique codant un activateur de transcription dHAP1 comme défini dans l'une quelconque des revendications 1 à 4, et une seconde séquence d'acide nucléique qui code un activateur de la transcription de GAL4;
(ii) introduire le premier gène d'intérêt dans une plante ou une partie de plante, ledit premier gène d'intérêt ayant une séquence d'activation amont répondant à HAP1; introduire le second gène d'intérêt dans une plante ou dans une partie de plante, ledit second gène d'intérêt ayant une séquence d'activation amont répondant à GAL4;

dans lequel la liaison dudit activateur de la transcription d'HAP1 à ladite séquence d'activation amont entraîne l'activation de la transcription du premier gène d'intérêt et la liaison dudit activateur de la transcription de GAL4 à ladite séquence d'activation amont provoque l'activation de la transcription du second gène d'intérêt.

37. Procédé pour coordonner l'expression d'une pluralité de gènes d'intérêt dans une plante ou partie de plante, comprenant les étapes consistant à:

(i) fournir une plante comprenant un acide nucléique codant un activateur de la transcription d'HAP1 comme défini dans l'une quelconque des revendications 1 à 4, et une seconde séquence d'acide nucléique, qui code un activateur de la transcription de GAL4;
(ii) introduire les gènes d'intérêt dans une plante ou une partie de plante, lesdits gènes d'intérêt étant chacun sous le contrôle d'une séquence d'activation amont répondant à HAP1,

dans lequel la liaison de l'activateur de la transcription d'HAP1 à ladite séquence d'activateur amont provoque l'activation de la transcription des gènes d'intérêt.

38. Procédé selon la revendication 37 dans lequel la pluralité de gènes sont associés à une seule séquence d'activateur amont.

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Figure 1

mHAP1-A

AAGCTTGGATCCAACAATGTCCTCCGACTCGTCCAAGATCAAGAGGAAGCGGAACCGCATCC

CGCTCAGCTGCACCATCTGCCGGAAGAGGAAGGTCAAGTGCGACAAGC

mHAP1-B

TCAGGCCGCACTGCCAGCAGTGCACCAAGACCGGGGTGGCCCACCTCTGCCACTACATGGAG

CAGACCTGGGCCGAGGAGGCCGAGAAGGAGTTGCTGAAGGACAACGAGTT

mHAP1-C

GAAGAAGCTCAGGGAGCGCGTGAAGTCCTTGGAGAAGACCCTCTCCAAGGTGCACTCCTCCC

CGTCGTCCAACTCCACGGCCCCCCCGACCGACGTCAGCCTGGGGGACGAGCTC

mHAP1-D

GGCAGTGCGGCCTGAGCTTGTCGCACTTGA

mHAP1-E

TCCCTGAGCTTCTTCAACTCGTTGTCCTTC

mHAP1-5'

CGGCAAGCTTGGATCCAACAATG

mHAP1-3'

CCCGGAGCTCGTCCCCCAGGCTG

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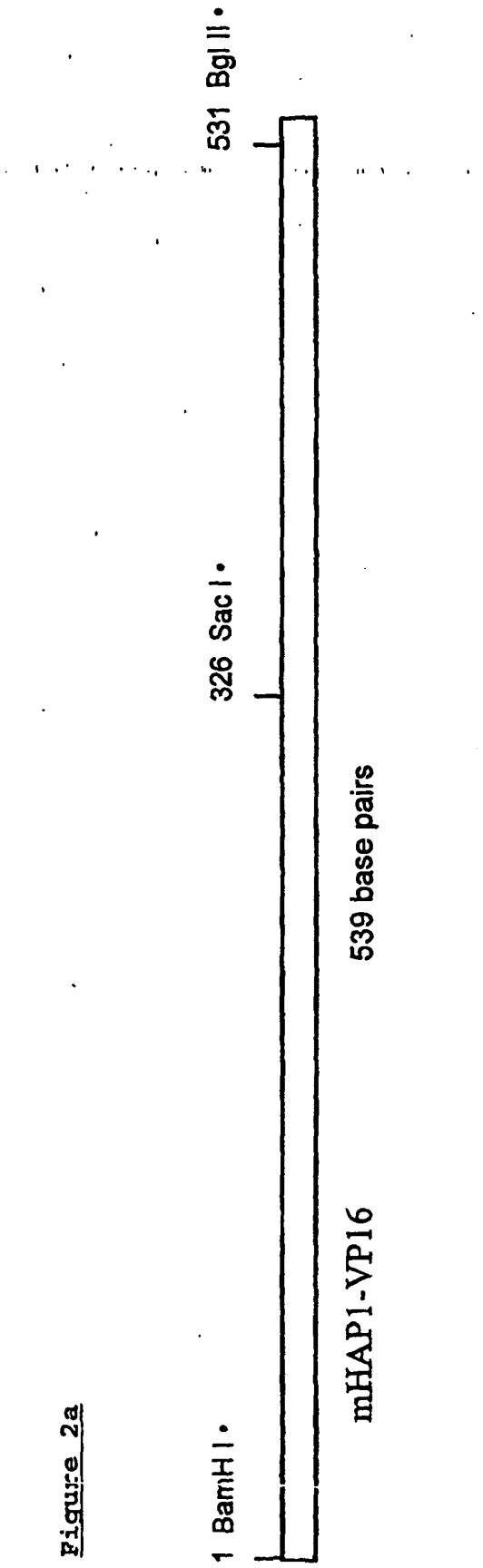

Figure 2a

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Figure 2b

1/1 47/11

atg tct tca gat tcg tcc aag atc aag agg aag cgt aac aga att ccg ctc agt tgc acc HAP1
atg tcC tcC gaC tcg tcc aag atc aag agg aag cgG aac CgC atC ccg ctc agC tgc acc mHAP1
M S S D S S K I K R K R N R I P L S C T

77/21 107/31

att tgt cgg aaa agg aaa gtc aaa tgt gac aaa ctc aga cca cac tgc cag cag tgc act HAP1
atC tgC cgg aaG agg aaG gtc aaG tgC gac aaG ctc agG ccG cac tgc cag cag tgc acC mHAP1
I C R K R K V K C D K L R P H C Q Q C T

137/41 167/51

aaa act ggg gta gcc cat ctc tgc cac tac atg gaa cag acc tgg gca gaa gag gca gag HAP1
aaG acC ggg gtG gcc caC ctc tgc cac tac atg gaG cag acc tgg gcC gaG gag gcC gag mHAP1
K T G V A H L C H Y M E Q T W A E E A E

197/61 227/71

aaa gaa ttg ctg aag gac aac gaa tta aag aag ctt agg gag cgc gta aaa tct tta gaa HAP1
aaG gaG ttg ctg aag gac aac gaG ttG aag aag ctC agg gag cgc gtG aaG tcC ttG gaG mHAP1
K E L L K D N E L K K L R E R V K S L E

257/81 287/91

aag act ctt tct aag gtg cac tct tct cct tcg tct aac tcc HAP1
aag acC ctC tcC aag gtg cac tcC tcC ccG tcg tcC aac tcc mHAP1
K T L S K V H S S P S S N S

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Figure 2c

11/1 41/11

atg tcC tcC gaC tcg tcc aag atc aag agg aag cgG aac CGC atC ccg ctc agC tgc acc

M S S D S S K I K R K R N R I P L S C T

71/21 101/31

atC tgC cgg aaG agg aaG gtc aaG tgC gac aaG ctc agG ccG cac tgc cag cag tgc acC

I C R K R K V K C D K L R P H C Q Q C T

131/41 161/51

aaG acC ggg gtG gcc caC ctc tgc cac tac atg gaG cag acc tgg gcC gaG gag gcC gag

K T G V A H L C H Y M E Q T W A E E A E

191/61 221/71

aaG gaG ttg ctg aag gac aac gaG ttG aag aag ctC agg gag cgc gtG aaG tcC ttG gaG

K E L L K D N E L K K L R E R V K S L E

251/81 281/91

aag acC ctC tcC aag gtg cac tcC tcC ccG tcg tcC aac tcc ACG GCC CCC CCG ACC GAC

K T L S K V H S S P S S N S T A P P T D

311/101 341/111

GTC AGC CTG GGG GAC GAG CTC CAC TTA GAC GGC GAG GAC GTG GCG ATG GCG CAT GCC GAC

V S L G D E L H L D G E D V A M A H A D

371/121 401/131

GCG CTA GAC GAT TTC GAT CTG GAC ATG TTG GGG GAC GGG GAT TCC CCG GGG CCG GGA TTT

A L D D F D L D M L G D G D S P G P G F

431/141 461/151

ACC CCC CAC GAC TCC GCC CCC TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT GAG CAG

T P H D S A P Y G A L D M A D F E F E Q

491/161 521/171

ATG TTT ACC GAT GCC CTT GGA ATT GAC GAG TAC GGT GGG TAG

M F T D A L G I D E Y G G *

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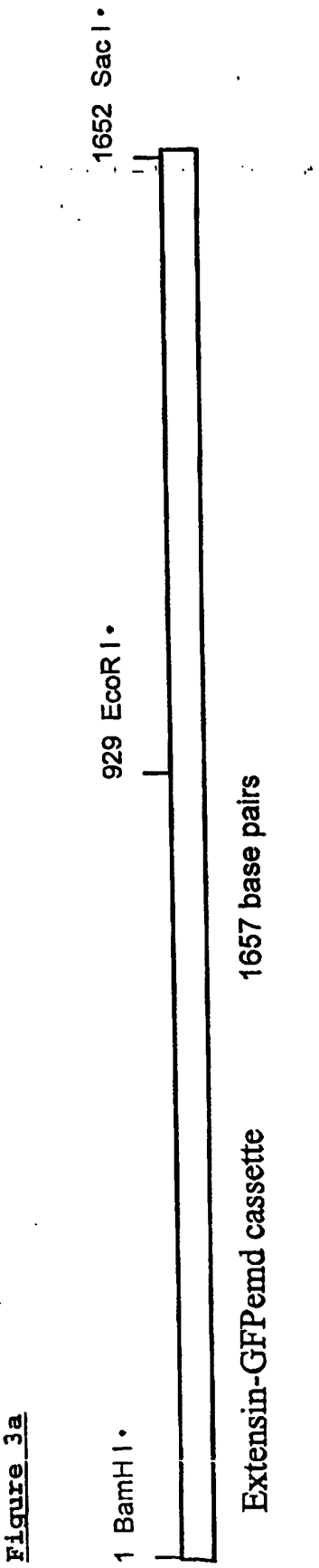

Figure 3a

Figure 3b

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11/1                                                41/11                                               71/21                                               101/31
atg gga aga att gct aga ggc tca aaa atg agt tct ctc att gtg tct ttg ctt gta gta ttg gtg tca ctc aat ttg gct tcc gaa acc aca gct aaa tac act tac tcc tct cca cca
M  G  R  I  A  R  G  S  K  M  S  S  L  I  V  S  L  L  V  V  L  V  S  L  N  L  A  S  E  T  T  A  K  Y  T  Y  S  S  P  P
131/41                                              161/51                                              191/61                                              221/71
cct ccc gag cat tct cct cca ccg ccg gag cat tct cct cct ccg cct tac cac tac gaa tcc ccg ccc ccg cct aaa cat tct cca cca cca cct aca ccg gtt tac aag tac aag tct
P  P  E  H  S  P  P  P  P  E  H  S  P  P  P  P  Y  H  Y  E  S  P  P  P  P  K  H  S  P  P  P  P  T  P  V  Y  K  Y  K  S
251/81                                              281/91                                              311/101                                             341/111
cca ccg cct cct atg cat tct cct cca ccg cct tat cat ttt gag tct cca cct cca cca aaa cat tct cca cca cca cca acg ccg gtt tac aag tac aaa tct cca cca cca cct aaa
P  P  P  P  M  H  S  P  P  P  P  Y  H  F  E  S  P  P  P  P  K  H  S  P  P  P  P  T  P  V  Y  K  Y  K  S  P  P  P  P  K
371/121                                             401/131                                             431/141                                             461/151
cat tct cct gca cca gtg cat cat tat aaa tac aag tct cca cca cca cca aca ccg gtt tat aag tat aaa tct cca cca cca cca aag cat tct cct gca cca gaa cat cac tat aag
H  S  P  A  P  V  H  H  Y  K  Y  K  S  P  P  P  P  T  P  V  Y  K  Y  K  S  P  P  P  P  K  H  S  P  A  P  E  H  H  Y  K
491/161                                             521/171                                             551/181                                             581/191
tac aag tct cca cca cca cct aag cat ttt cct gca cca gaa cat cac tat aag tac aag tac aag tct cca cca cca cca aca ccg gtc tac aag tat aaa tct cca cca cct cca aca
Y  K  S  P  P  P  P  K  H  F  P  A  P  E  H  H  Y  K  Y  K  Y  K  S  P  P  P  P  T  P  V  Y  K  Y  K  S  P  P  P  P  T
611/201                                             641/211                                             671/221                                             701/231
ccg gtc tac aag tac aag tct cca cca cca ccc aag cat tct ccc gca cca gta cac cat tac aag tac aag tct cca cca cca cca act cca gtt tat aaa tct cca cca cca ccc gaa
P  V  Y  K  Y  K  S  P  P  P  P  K  H  S  P  A  P  V  H  H  Y  K  Y  K  S  P  P  P  P  T  P  V  Y  K  S  P  P  P  P  E
731/241                                             761/251                                             791/261                                             821/271
cat tcc cca cca cca cca aca ccg gtc tac aaa tac aag tct cca cca cca cca atg cac tct cca cca cca cca aca cca gtt tac aag tac aag tct ccg cca cca cca atg cac tct
H  S  P  P  P  P  T  P  V  Y  K  Y  K  S  P  P  P  P  M  H  S  P  P  P  P  T  P  V  Y  K  Y  K  S  P  P  P  P  M  H  S
851/281                                             881/291                                             911/301                                             941/311
ccc cca cca cca gtt tac tct cca cca cca ccc aaa cat cac tac tcc tat acg tca cct cct cct cct cac cac tac gaa ttc GTG AGC AAG GGC GAG GAG CTG TTC ACC GGG GTG GTG
P  P  P  P  V  Y  S  P  P  P  P  K  H  H  Y  S  Y  T  S  P  P  P  P  H  H  Y  E  F  V  S  K  G  E  E  L  F  T  G  V  V
971/321                                             1001/331                                            1031/341                                            1061/351
CCC ATC CTG GTC GAG CTG GAC GGC GAC GTA AAC GGC CAC AAG TTC AGC GTG TCC GGC GAG GGC GAG GGC GAT GCC ACC TAC GGC AAG CTG ACC CTG AAG TTC ATC TGC ACC ACC GGC AAG
P  I  L  V  E  L  D  G  D  V  N  G  H  K  F  S  V  S  G  E  G  E  G  D  A  T  Y  G  K  L  T  L  K  F  I  C  T  T  G  K
1091/361                                            1121/371                                            1151/381                                            1181/391
CTG CCC GTG CCC TGG CCC ACC CTC GTG ACC ACC TTG ACC TAC GGC GTG CAG TGC TTC GCC CGC TAC CCC GAC CAC ATG AAG CAG CAC GAC TTC TTC AAG TCC GCC ATG CCC GAA GGC TAC
L  P  V  P  W  P  T  L  V  T  T  L  T  Y  G  V  Q  C  F  A  R  Y  P  D  H  M  K  Q  H  D  F  F  K  S  A  M  P  E  G  Y
1211/401                                            1241/411                                            1271/421                                            1301/431
GTC CAG GAG CGC ACC ATC TTC TTC AAG GAC GAC GGC AAC TAC AAG ACC CGC GCC GAG GTG AAG TTC GAG GGC GAC ACC CTG GTG AAC CGC ATC GAG CTG AAG GGC ATC GAC TTC AAG GAG
V  Q  E  R  T  I  F  F  K  D  D  G  N  Y  K  T  R  A  E  V  K  F  E  G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E
1331/441                                            1361/451                                            1391/461                                            1421/471
GAC GGC AAC ATC CTG GGG CAC AAG CTG GAG TAC AAC TAC AAC AGC CAC AAG GTC TAT ATC ACC GCC GAC AAG CAG AAG AAC GGC ATC AAG GTG AAC TTC AAG ACC CGC CAC AAC ATC GAG
D  G  N  I  L  G  H  K  L  E  Y  N  Y  N  S  H  K  V  Y  I  T  A  D  K  Q  K  N  G  I  K  V  N  F  K  T  R  H  N  I  E
1451/481                                            1481/491                                            1511/501                                            1541/511
GAC GGC AGC GTG CAG CTC GCC GAC CAC TAC CAG CAG AAC ACC CCC ATC GGC GAC GGC CCC GTG CTG CTG CCC GAC AAC CAC TAC CTG AGC ACC CAG TCC GCC CTG AGC AAA GAC CCC AAC
D  G  S  V  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L  L  P  D  N  H  Y  L  S  T  Q  S  A  L  S  K  D  P  N
1571/521                                            1601/531                                            1631/541
GAG AAG CGC GAT CAC ATG GTC CTG CTG GAG TTC GTG ACC GCC GCC GGG ATC ACT CTC GGC ATG GAC GAG CTG TAC AAG TAA
E  K  R  D  H  M  V  L  L  E  F  V  T  A  A  G  I  T  L  G  M  D  E  L  Y  K  *
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Patent Lens
enabling INNOVATION

Patent Lens
enabling INNOVATION

Figure 4

Patent Lens
enabling INNOVATION

Figure 5